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(57) Abstract

The present invention provides recombinant or chimaeric DNA molecules comprising plant SAR genes, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis independent or in a protein-synthesis dependent manner. Methods for obtaining these DNA molecules are also provided. Additionally, the present invention provides chemically inducible wheat genes, Arabidopsis chitinase IV, Maize PR-1mz, and Maize thaumatin PR-5mz. The anti-pathogenic sequences according to the invention can be genetically engineered and transformed into plants to obtain transgenic plants with enhanced resistance to disease. In one embodiment of the present invention, the chemically regulatable DNA promoter sequence of the Arabidopsis Pr-1 gene is provided. Another aspect of the present invention is a method of improving protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins exert a synergistic effect.

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# *CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES AND USES THEREOF*

The present invention relates to DNA molecules encoding proteins capable of conferring plant disease and/or plant pest resistance. Another aspect of the invention relates to the chemical regulation of gene expression. In particular, this invention relates to non-coding DNA sequences which, in the presence of chemical regulators, regulate the transcription of associated DNA sequences in plants. Both aspects of the invention relate, in part, to genes associated with the response of plants to pathogens.

Advances in recombinant DNA technology coupled with advances in plant transformation and regeneration technology have made it possible to introduce new genetic material into plant cells, plants or plant tissue, thus introducing new traits, e.g., phenotypes, that enhance the value of the plant or plant tissue. Recent demonstrations of genetically engineered plants resistant to pathogens (EP-A 240 332 and EP-A 223 452) or insects (Vaeck, M. *et al., Nature 328:* 33 (1987)) and the production of herbicide tolerant plants (DeBlock, M. *et al., EMBO J. 6:* 2513 (1987)) highlight the potential for crop improvement. The target crops can range from trees and shrubs to ornamental flowers and field crops. Indeed, it is clear that the "crop" can also be a culture of plant tissue grown in a bioreactor as a source for some natural product.

Various methods are known in the art to accomplish the genetic transformation of plants and plant tissues (i.e., the stable introduction of foreign DNA into plants). These include transformation by Agrobacterium species and transformation by direct gene transfer.

A. tumefaciens is the etiologic agent of crown gall, a disease of a wide range of dicotyledons and gymnosperms, that results in the formation of tumors or galls in plant tissue at the site of infection. Agrobacterium, which normally infects the plant at wound sites, carries a large extrachromosomal element called the Ti (tumor-inducing) plasmid.

Ti plasmids contain two regions required for tumorigenicity. One region is the T-DNA (transferred-DNA) which is the DNA sequence that is ultimately found stably transferred to plant genomic DNA. The other region required for tumorigenicity is the vir (virulence) region which has been implicated in the transfer mechanism. Although the vir region is absolutely required for stable transformation, the vir DNA is not actually transferred to the infected plant. Transformation of plant cells mediated by infection with Agrobacterium tumefaciens

and subsequent transfer of the T-DNA alone have been well documented. See, for example, Bevan, M.W. and Chilton, M-D., *Int. Rev. Genet. 16:* 357 (1982).

After several years of intense research in many laboratories, the Agrobacterium system has been developed to permit routine transformation of a variety of plant tissue. Representative species transformed in this manner include tobacco, tomato, sunflower, cotton, rapeseed, potato, soybean, and poplar. While the host range for Ti plasmid transformation using A. tumefaciens as the infecting agent is known to be very large, tobacco has been a host of choice in laboratory experiments because of its ease of manipulation.

Agrobacterium rhizogenes has also been used as a vector for plant transformation. This bacterium, which incites hairy root formation in many dicotyledonous plant species, carries a large extrachromosomal element called an Ri (root-inducing) plasmid which functions in a manner analogous to the Ti plasmid of A. tumefaciens. Transformation using A. rhizogenes has developed analogously to that of A. tumefaciens and has been successfully utilized to transform, for example, alfalfa, Solanum nigrum L., and poplar.

Several so-called direct gene transfer procedures have been developed to transform plants and plant tissues without the use of an Agrobacterium intermediate (see, for example, Koziel *et al., Biotechnology 11:* 194-200 (1993) herein incorporated by reference). In the direct transformation of protoplasts the uptake of exogenous genetic material into a protoplast may be enhanced by use of a chemical agent or electric field. The exogenous material may then be integrated into the nuclear genome. The early work was conducted in the dicot tobacco where it was shown that the foreign DNA was incorporated and transmitted to progeny plants, see e.g. Paszkowski, J. *et al., EMBO J. 3:* 2717 (1984); and Potrykus, I. *et al., Mol. Gen. Genet. 199:* 169 (1985).

Monocot protoplasts have also been transformed by this procedure in, for example, Triticum monococcum, Lolium multiflorum (Italian ryegrass), maize, and Black Mexican sweet corn.

Alternatively exogenous DNA can be introduced into cells or protoplasts by microinjection. A solution of plasmid DNA is injected directly into the cell with a finely pulled glass needle. In this manner alfalfa protoplasts have been transformed by a variety of plasmids, see e.g. Reich, T.J. *et al., Bio/Technology 4:* 1001 (1986).

A more recently developed procedure for direct gene transfer involves bombardment of cells by microprojectiles carrying DNA, see Klein, T.M. *et al., Nature 327:* 70 (1987). In this procedure tungsten particles coated with the exogenous DNA are accelerated toward the target cells, resulting in at least transient expression in the example reported (onion).

Just as there is a variety of methods for the transformation of plant tissue, there is a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated. In recent years it has become possible to regenerate many species of plants from callus tissue derived from plant explants. The plants which can be regenerated from callus include monocots, such as corn, rice, barley, wheat and rye, and dicots, such as sunflower, soybean, cotton, rapeseed and tobacco.

Regeneration of plants from tissue transformed with A. tumefaciens has been demonstrated for several species of plants. These include sunflower, tomato, white clover, rapeseed, cotton, tobacco, and poplar. The regeneration of alfalfa from tissue transformed with A. rhizogenes has also been demonstrated. Plant regeneration from protoplasts is a particularly useful technique, see Evans, D.A. *et al.*, in: "Handbook of Plant Cell Culture", Vol. 1, MacMillan Publ. Co., 1983, p. 124. When a plant species can be regenerated from protoplasts, then direct gene transfer procedures can be utilized, and transformation is not dependent on the use of A. tumefaciens. Regeneration of plants from protoplasts has been demonstrated for rice, tobacco, rapeseed, potato, eggplant, cucumber, poplar, and corn.

Various plant tissues may be utilized for transformation with foreign DNA. For instance, cotyledon shoot cultures of tomato have been utilized for Agrobacterium mediated transformation and regeneration of plants (see European application EP-A 249432). Further examples include Brassica species (see WO 87/07299) and woody plant species, particularly poplar (see U.S. Patent No. 4,795,855, incorporated by reference herein in its entirety).

The technological advances in plant transformation and regeneration technology highlight the potential for crop improvement via genetic engineering. There have been reports of genetically engineered tobacco and tomato plants which are resistant to infections of, for example, tobacco mosaic virus (TMV) and resistant to different classes of herbicides. Insect resistance has been engineered in tobacco and tomato plants.

The potential for genetic engineering is not limited to field crops but includes improvements in ornamentals, forage crops and trees. A less obvious goal for plant biotechnology, which includes both genetic engineering and tissue culture applications, is the enhanced production of a vast array of plant-derived chemical compounds including flavors, fragrances, pigments, natural sweeteners, industrial feedstocks, antimicrobials and pharmaceuticals. In most instances these compounds belong to a rather broad metabolic group, collectively denoted as secondary products. Plants may produce such secondary products to ward off potential predators, attract pollinators, or combat infectious diseases.

Plant cell cultures can be established from an impressive array of plant species and may be propagated in a bioreactor. Typical plant species include most of those that produce secondary products of commercial interest. It has been clearly demonstrated in a number of agriculturally important crop plants that stable genetic variants arising from the tissue culture of plant somatic cells (somaclonal variation) can be induced and selected. Numerous advantages flow from plant tissue culture production of secondary compounds. These include (1) the possibility of increased purity of the resultant product, (2) the conversion of inexpensive precursors into expensive end products by biotransformation, and (3) the potential for feeding substrate analogs to the culture to create novel compounds.

Whether the target of genetic engineering of plants is a field crop, ornamental shrub, flower, tree or a tissue culture for use in a bioreactor, a principal advantage to be realized is the control of expression of the chimeric gene so that it is expressed only at the appropriate time and to the appropriate extent, and in some situations in particular parts of the plant. For example, in order to achieve a desirable phenotype the chimeric gene may need to be expressed at levels of 1% of the total protein or higher. This may well be the case for fungal resistance due to chimeric chitinase expression or insect resistance due to increased proteinase inhibitor expression. In these cases the energy expended to produce high levels of the foreign protein may result in a detriment to the plant whereas, if the gene were expressed only when desired, for instance when a fungal or insect infestation is imminent, the drain on energy, and therefore yield, could be reduced.

Alternatively, the phenotype expressed by the chimeric gene could result in adverse effects to the plant if expressed at inappropriate times during development. For example, if the chimeric gene product were a plant hormone that induced pod abscission, early expression could bring about abscission of the fruit from the plant before the seed had

matured, resulting in decreased yield. In this case it would be much more advantageous to induce the expression of this type of gene to a time when pod abscission is preferred, or least injurious to the plant.

For tissue in culture or in a bioreactor the untimely production of a secondary product could lead to a decrease in the growth rate of the culture resulting in a decrease in the yield of the product. Therefore, it would be advantageous to allow the culture to grow without expressing the secondary product and then induce the chimeric gene at an appropriate time to allow for an optimized expression of the desired product.

In view of considerations like these, as well as others, it is clear that control of the time, extent and/or site of expression of the chimeric gene in plants or plant tissues would be highly desirable. Control that could be exercised easily in a field, a greenhouse or a bioreactor would be of particular commercial value.

External manipulation of the expression of endogenous genes which contain chemically regulatable sequences by the application of a chemical regulator is already known from Ward, E. *et al., Plant Cell 3:* 1085-1094 (1991); Williams *et al., Bio/Technology 10:* 540-543 (1992); and Uknes, S. *et al., Plant Cell 5:* 159-169 (1993). The control provided is somewhat limited, however, due to the responsiveness of such sequences to endogenous chemical metabolites and cell signals as well as externally applied chemical regulators. In one aspect of the invention, alterations are taught which block the responsiveness of these genes to endogenous signals while maintaining responsiveness to externally applied chemical regulators.

Pest infestation of crop plants causes considerable loss of yield throughout the world and most crops grown in the U.S. suffer infestation, particularly from insects. Major insect pests in the U.S. include the European Corn Borer (*Ostrinia nubilalis*) in maize, the cotton bollworm (*Heliothis zea*) and the pink bollworm (*Pectinophora gossypiella*) in cotton and the tobacco budworm (*Heliothis virescens*) in tobacco. Resistance to pests is ddifficult to achieve using conventional breeding programs and typically pests have been controlled using chemical pesticides.

Recent advances in molecular biology and plant transformation technology have demonstrated the possibility of expressing in transgenic plants genes encoding insecticidal proteins; this represents a novel approach in the production of crop plants resistant to

pests. Most notably, the expression of genes encoding the *Bacillus thuringiensis* endotoxin has been successful in a wide range of plant species, and the analysis of transgenic lines expressing such genes has been well documented (Vaeck *et al., Nature 328:* 33-37 (1987); Fischoff *et al., Biotechnology 5:* 807-813 (1987); Carozzi *et al., Plant Mol. Biol. 20:* 539-548 (1992); Koziel *et al., Biotechnology 11:* 194-200 (1993)). Other insecticidal genes have been used successfully in generating insect resistant transgenic plants.

One approach has been the overexpression of genes encoding insect enzyme inhibitors such as trypsin inhibitors or seed proteins with known insecticidal activity, such as lectins (Hilder *et al., Nature 330:* 160-163 (1987)). Indeed, plants expressing both the cowpea trypsin inhibitor and pea lectin were shown to have additive effects in providing insect resistance (Boulter *et al., Crop Protection 9:* 351-354 (1990)). In cases where pests are able infest parts of the plant or tissues not readily accessible to conventional pesticides, a transgenic approach may be more successful than the use of conventional pesticides.

For example, the tobacco budworm *Heliothis* is well known to be difficult to control using pesticides because it burrows deep into the plant tissue. Additionally, some pests of roots, such as nematodes, are not readily controlled by foliar applications of pesticides. An advantage in the use of transgenic plants expressing insecticidal proteins is the controlled expression of the proteins in all desired tissues.

Chitinases catalyze the hydrolysis of chitin, a ß-1,4-linked homopolymer of *N*-acetyl-D-glucosamine. Several different plant chitinases have been described and the cDNA sequences for some of these have been reported (Meins *et al., Mol. Gen. Genet. 232:* 460-469 (1992)). Based on structural characteristics, three classes have been distinguished. Class I chitinases have two structural domains, a cysteine-rich amino-terminal hevein domain and a carboxyterminal catalytic domain separated from the former by a variable spacer. Class II chitinases lack the cysteine-rich hevein domain and all or part of the variable spacer, but retain the catalytic domain. Class III chitinases lack the hevein domain and contain a catalytic domain that shares no significant homology with that of the class I or class II enzymes.

Class I chitinase gene expression is induced by ethylene, whereas class II and class III chitinase gene expression is induced in the SAR response. The chitinase/lysozyme disclosed in European patent application EP-A 392 225 and the chitinase/lysozyme disclosed in SEQ ID Nos. 20 and 21 are class III chitinases. It is well known that the level of chitinase activity of plants increases dramatically after pathogen invasion (Mauch *et al.,*

*Plant Physiol. 76:* 607-611 (1984)) and this is presumably due to the host plant's attempts to degrade the chitin of the fungal cell wall. Furthermore, chitinase has been shown *in vitro* to inhibit fungal and insect growth, and in transgenic plants a bacterial chitinase has been shown to exhibit inhibitory effects towards numerous pathogens and pests including insects (Suslow & Jones WPO 90-231246; U.S. Pat. Nos. 4,940,840 and 4,751,081; herein incorporated by reference in their entirety).

For over 90 years, scientists and naturalists have observed that when plants survive pathogen infection they develop an increased resistance to subsequent infections. In 1933, a phenomenon termed "physiological acquired immunity" was described in an extensive literature review by Chester, K.S., *Quart. Rev. Biol. 8:* 275-324 (1933). At that time, scientists believed they were investigating a phenomenon analogous to the immune response in mammals. In retrospect, at least three different processes were being called "aquired immunity": viral cross protection, antagonism (or biocontrol), and what we now refer to as systemic acquired resistance (SAR).

The first systematic study of SAR was published by A. Frank Ross in 1961. Using tobacco mosaic virus (TMV) on local lesion hosts, Ross demonstrated that infections of TMV were restricted by a prior infection. This resistance was effective against not only TMV, but also tobacco necrosis virus and certain bacterial pathogens. Ross coined the term "systemic acquired resistance" to refer to the inducible systemic resistance (Ross, A.F., *Virology 14:* 340-358 (1961)) and "localized acquired resistance" (LAR) to describe the resistance induced in inoculated leaves (Ross, A.F., *Virology 14:* 329-339 (1961)). It is still unclear whether SAR and LAR are aspects of the same response or distinct processes.

In the past 30 years, SAR has been demonstrated in many plant species and the spectrum of resistance has been broadened to include not only viruses and bacteria, but also many agronomically important phytopathogenic fungi (*see* Kuc, J., *BioScience 32:* 854-860 (1982). However, understanding of the biochemical events leading to the establishment of SAR had not progressed substantially until the past dozen years. In 1982, the accumulation of a group of extracellular proteins called pathogenesis-related (PR) proteins were reported to correlate with the onset of SAR (Van Loon, L.C. *et al., Neth. J. Plant. Path. 88:* 237-256 (1982)). In 1979, salicylic acid (SA) and certain benzoic acid derivatives were reported to be able to induce both resistance and the accumulation of PR proteins (White,

R.F., *Virology 99:* 410-412 (1979). As a result, SA was considered as a possible endogenous signal molecule (Van Loon, L.C. *et al., Neth. J. Plant. Path. 88:* 237-256 (1982)). However, progress slowed through the 1980's and the involvement of PR proteins and salicylic acid in SAR was questioned.

With the advent of genetic engineering and recombinant DNA technology, the possibility of manipulating genetic material to improve the phenotype of plants has arisen. The present invention is based in part upon the discovery of the identity and role of genes involved in SAR which has allowed the application of modern molecular biological techniques for improved plant disease and plant pest resistance.

There are two major aspects of the present invention. The first aspect relates to chemically regulatable DNA sequences and the chemicals which regulate them. The second aspect relates to plant pathogenesis-related proteins. Both aspects of the invention have arisen, in part, from the inventors' identification and characterization of cDNAs and corresponding genes involved in the plant response to pathogen infection.

A principal object of the present invention is to provide additional means for chemically regulating the expression of a desired gene in a plant, seed, or plant tissue. In particular, the present invention relates to identification and isolation of a non-coding DNA sequence obtainable from the promoter region of an *Arabidopsis* PR-1a gene which, in the presence of chemical regulators, regulate the transcription of associated DNA sequences in plants.

Another principal object of the present invention is to provide transgenic plants, transgenic seeds and the progeny thereof expressing levels of plant pathogenesis-related anti-pathogenic proteins such as tobacco chitinase/lysozyme according to the invention, or substantially homologous proteins, which confer an enhanced disease-resistant and/or pest-resistant phenotype with respect to wild-type plants.

Accordingly, to meet this objective and others, the second aspect of the present invention disclosed herein provides for the isolation, cloning and identification of novel cDNA clones coding for plant pathogenesis-related (PR) proteins. These cDNAs, or their genomic counterparts, or DNA molecules with substantial homology to either (all of the above collectively referred to herein as "anti-pathogenic sequences"), can be engineered for expression of the encoded PR proteins or anti-sense RNA and transformed into plants to confer enhanced resistance or tolerance to various diseases and/or pests as described

herein. These DNA molecules may be engineered for constitutive expression, expression in particular tissues or at certain developmental stages, or induced expression in response to an inducer, for example in response to a chemical inducer as described herein.

The present invention further provides novel methods for differential screening and enriching for induced cDNAs, particularly those cDNAs induced in response to pathogen infection or a chemical inducer which triggers a response mimicking pathogen infection.

The present invention teaches the cloning of SAR genes by differential screening of tissues induced and non-induced to the systemic acquired response. SAR induction was found cause the transcription of genes in a protein synthesis-dependent fashion and also a protein synthesis-independent fashion. Two methods were used to clone specifically genes whose induced transcription is protein-synthesis independent:

Firstly, recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis independent manner, are obtained by

(a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
(b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
(c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

Thus, cDNAs which were cloned by standard differential screening techniques were further screened on SAR-induced RNA isolated with and without cycloheximide (CHX) pre-treatment.

Secondly, recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis independent manner, are obtained by

(a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;

(b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
(c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

Thus, a PCR-based "differential display" technique is used to identify SAR-induced, but protein synthesis independent cDNAs directly.

Differential display RNAs were prepared with and without SAR induction and CHX treatment. CHX as an inhibitor of protein synthesis is well known in the art and described by Greenberg *et al., Mol. Cell Biol. 6:* 1050-1057 (1986), Lau and Nathans, *Proc. Natl. Acad. Sci. 84:* 1182-1186 (1987), and Uknes *et al., Plant Cell 5:* 159-169 (1993). The methods described provide recombinant or chimaeric DNA molecules comprising plant SAR genes and preferably plant SAR cDNA molecules, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis independent manner, that is in the presence of a protein synthesis inhibitor such as cycloheximide. Thus, a number of genes were cloned which were induced by the SAR response, yet expressed independently of protein synthesis. These cloned genes are likely signal transducers in the pathway leading from induction to the development of the resistant state.

Additionally recombinant or chimaeric DNA molecules are provided which comprise plant SAR genes and preferably plant SAR cDNA molecules, wherein the wild-type genes corresponding to said SAR genes can be chemically induced in a plant in a protein-synthesis dependent manner, that is only in the absence of a protein synthesis inhibitor such as cycloheximide. Recombinant or chimaeric DNA molecules encoding genes which correspond to wild-type genes which can be chemically induced in a protein-synthesis dependent manner, can be obtained by

(a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
(b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor such as cycloheximide; and

(c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

Alternatively, these molecules can be obtained by

(a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
(b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor such as cycloheximide; and
(c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

***DESCRIPTION OF GENE SEQUENCES***

SEQ ID No. 1: The cDNA sequence encoding an *Arabidopsis* class IV chitinase with a hevein domain (pChit4-TA).

SEQ ID No. 2: The cDNA sequence encoding an *Arabidopsis* class IV chitinase without a hevein domain (pChit4-TB).

SEQ ID No. 3: The cDNA sequence of the wheat gene WCI-1.

SEQ ID No. 4: The partial cDNA sequence of the 5′ end of the wheat gene WCI-2 which encodes a lipoxygenase isozyme. The partial sequence of the 3′ end of this cDNA is provided in SEQ ID No. 5.

SEQ ID No. 5: The partial cDNA sequence of the 3′ end of the wheat gene WCI-2 which encodes a lipoxygenase isozyme. The partial sequence of the 5′ end of this cDNA is provided in SEQ ID No. 4.

SEQ ID No. 6: The cDNA sequence of the wheat gene WCI-3.

SEQ ID No. 7: The cDNA sequence of the wheat gene WCI-4.

SEQ ID No. 8: The cDNA sequence of the wheat gene WCI-5.

SEQ ID No. 9: A tobacco protein-synthesis dependent gene involved in the regulation of the systemic acquired resistance response designated p1.1.1.

SEQ ID No. 10: A tobacco protein-synthesis dependent gene involved in the regulation of the systemic acquired resistance response designated p11.31.4.

SEQ ID No. 11: The 5' DNA sequence of a tobacco protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated p11.30.13.

SEQ ID No. 12: The DNA sequence of the 3'end of the same protein-synthesis independent gene described in Seq. ID. No. 11 cloned from tobacco and involved in the regulation of the systemic acquired resistance response designated p11.30.13. This sequence is derived from the non-coding strand (*i.e.* the "bottom" strand). The first base listed is therefore located in the furthest 3' position.

SEQ ID No. 13: A tobacco protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated p1.4.3. This sequence is identical to the thioredoxin gene published by Brugidou *et al., Mol. Gen. Genet. 238:* 285-293 (1993).

SEQ ID No. 14: A protein-synthesis independent SAR gene cloned from tobacco designated p66B1.

SEQ ID No. 15: A protein-synthesis dependent SAR gene cloned from tobacco designated p14.22.3.

SEQ ID No. 16: An *Arabidopsis* protein-synthesis independent gene involved in the regulation of the systemic acquired resistance response designated pDPA2.

SEQ ID No. 17: The cDNA sequence encoding an Arabidopsis PR-1 protein cloned into plasmid pAPR1C-1.

SEQ ID No. 18: Oligo B used in example 4A

SEQ ID No. 19: Oligo C used in example 4A

SEQ ID No. 20: The cDNA sequence encoding a basic tobacco chitinase/lysozyme protein cloned into the plasmid pBSCL2.

SEQ ID No. 21: The cDNA sequence encoding an acidic tobacco chitinase/lysozyme protein cloned into the plasmid pBSTCL226.

SEQ ID No. 22: A representative molecular adaptor sequence.

SEQ ID No. 23: Oligonucleotide primer for the PR-1 gene.

SEQ ID No. 24: Oligonucleotide primer for GUS gene.

SEQ ID No. 25: Oligonucleotide primer for the AHAS gene.
SEQ ID No. 26: Oligonucleotide primer for the BT gene.
SEQ ID No. 27: Peptide sequence used to design a degenerate forward oligonucleotide sequence for PCR cloning of chitinase IV
SEQ ID No. 28: Peptide sequence used to design a degenerate reverse oligonucleotide sequence for PCR cloning of chitinase IV
SEQ ID No. 29: The cDNA sequence of the Maize PR1-like gene PR-1mz
SEQ ID No. 30: Amino acid sequence of the protein encoded by SEQ ID No. 29
SEQ ID No. 31: The cDNA sequence of the Maize thaumatin-like gene PR-5mz
SEQ ID No. 32: Amino acid sequence of the protein encoded by SEQ ID No. 31
SEQ ID No. 33: The cDNA sequence of the Arabidopsis gene PSI-1
SEQ ID No. 34: The cDNA sequence of the Arabidopsis gene PSI-2
SEQ ID No. 35: The cDNA sequence of the Arabidopsis gene PSI-3
SEQ ID No. 36: The cDNA sequence of the Arabidopsis gene PSI-4
SEQ ID No. 37: The cDNA sequence of the Arabidopsis gene PSI-5

***DEFINITIONS***

In order to provide a clear and consistent understanding of the specification and the claims, including the scope given to such terms, the following definitions are provided:

Anti-pathogenic Sequence: A DNA molecule encoding a plant pathogenesis-related (PR) protein, or a DNA molecule with substantial homology thereto, which is capable of conferring enhanced resistance or tolerance to disease and/or pests when expressed in a plant, seed, or plant tissue.

Anti-sense Mechanism: A mechanism for regulation of gene expression based on the presence in a cell of a RNA molecule complementary to at least a portion of the mRNA encoded by the gene. This mechanism is thought to involve controlling the rate of translation of mRNA to protein due to the presence in a cell of an RNA molecule complementary to at least a portion of the mRNA being translated.

Associated DNA Sequence: A DNA sequence whose cellular activity either (I) regulates the activity of another DNA sequence or (2) is regulated by another DNA sequence. This definition specifically embraces, but is not limited to, sequences which are physically adjacent in a continuous DNA strand or which are physically separated. Physical separation includes, for example, separation within the same DNA strand, location within different DNA strands, or discontinuous interspersed sequences (e.g., alternating regulatable and coding sequences) in one strand.

Chemically Regulatable DNA Sequence: A DNA sequence which is capable of regulating the transcription of an associated DNA sequence where the regulation is dependent on a chemical regulator. The sequences may be of natural or synthetic origin.

Chemically Regulatable Gene: A gene containing at least one non-coding chemically regulatable DNA sequence and at least one associated coding DNA sequence. The genes may be of natural, synthetic or partially natural/partially synthetic origin.

Chemical Regulator (for a chemically regulatable DNA sequence): An elemental or molecular species which controls (e.g., initiates, terminates, increases or reduces), by direct or indirect action, the activity of a chemically regulatable DNA sequence in a system in which the chemical regulator is not normally found in an active form in an amount sufficient to effect regulation of transcription, to the degree and at the time desired, of a transcribable DNA sequence associated with the chemically regulatable DNA sequence. This terminology is intended to embrace situations in which no or very little regulator is present at the time transcription is desired or in which some regulator is present but increased or decreased regulation is required to effect more or less transcription as desired.

Thus, if the system containing the chemically regulatable DNA sequence is a plant, for example a transgenic plant, a chemical regulator is a species not naturally found in the plant in an amount sufficient to effect chemical regulation, and thus transcription of an associated gene, to the desired degree at the time desired.

By "direct action" it is intended that the chemical regulator action result from the direct interaction between the chemical regulator and the DNA sequence. By "indirect action" it is meant that the regulator action results from the direct interaction between the chemical regulator and some other endogenous or exogenous component in the system,

the ultimate result of that direct interaction being activation or suppression of the activity of the DNA sequence. By "active form" it is intended that the chemical regulator be in a form required to effect control.

Chimeric Sequence or Gene: A DNA sequence containing at least two heterologous parts, e.g., parts derived from naturally occurring DNA sequences which are not associated in their naturally occurring states, or containing at least one part that is of synthetic origin and not found in nature.

Coding DNA Sequence: A DNA sequence which, when transcribed and translated, results in the formation of a cellular polypeptide.

Constitutive transcription: Transcription of substantially fixed amounts of a DNA sequence, irrespective of environmental conditions.

Gene: A discrete chromosomal region which is responsible for a discrete cellular product.
Inducers: Molecules that cause the production of larger amounts of macromolecules, compared to the amounts found in the absence of the inducer.

Inducible Protein: Proteins whose rate of production can be increased by the presence of inducers in the environment.

Non-coding DNA Sequence: A DNA sequence, which is not transcribed and translated, resulting in the formation of a cellular polypeptide when associated with a particular coding DNA sequence. A sequence that is non-coding when associated with one coding sequence may actually be coding when associated with another coding or non-coding sequence.

Phenotypic Trait: An observable property resulting from expression of a gene.

Plant Tissue: Any tissue of a plant in planta or in culture. This term includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures and any groups of plant cells organized into structural and/or functional units. The

use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

PR, or Pathogenesis-Related Proteins: Proteins expressed in plants reacting hypersensitively towards pathogens. This term embraces, but is not limited to, SAR8.2a and SAR8.2b proteins, the acidic and basic forms of tobacco PR-1a, PR-1b, PR-1c, PR-1′, PR-2, PR-2′, PR-2", PR-N, PR-O, PR-O′, PR-4, PR-P, PR-Q, PR-S, and PR-R major proteins, cucumber peroxidases, basic cucumber peroxidase, the chitinase which is a basic counterpart of PR-P or PR-Q, and the beta-1,3-glucanase (glucan endo-1,3-ß-glucosidase, EC 3.2.1.39) which is a basic counterpart of PR-2, PR-N or PR-O, the pathogen-inducible chitinase from cucumber, class IV chitinases with and without a hevein domain, and the WCI ("Wheat Chemically Induced") gene proteins from . A hypersensitive reaction is characterized by a local necrosis of the tissues immediately surrounding the infection site of the pathogen and a subsequent localization of the pathogen, which is in contrast to a sensitive reaction wherein the pathogen spreads throughout the plant. Pathogens are, for example, viruses or viroids, e.g. tobacco or cucumber mosaic virus, ringspot virus or necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato bushy stunt virus, and like viruses, fungi, e.g. Phythophthora parasitica or Peronospora tabacina, bacteria, e.g. Pseudomonas syringae or Pseudomonas tabaci, or aphids, e.g. Myzus persicae. This list is not limiting in any respect.

Regulation: The increasing (inducing) or decreasing (repressing) of the level of expression of a gene or the level of transcription of a DNA sequence. The definition is not intended to embrace any particular mechanism.

Substantially Pure DNA Sequence: A DNA molecule (sequence) isolated in substantially pure form from a natural or non-natural source. Such a molecule may occur in a natural system, for example, in bacteria, viruses or in plant or animal cells, or may be provided, for example, by synthetic means or as a cDNA. Substantially pure DNA sequences are typically isolated in the context of a cloning vector. Substantially pure means that DNA sequences other than the ones intended are present only in marginal amounts, for example less than 5%, less than 1%, or preferably less than 0.1%. Substantially pure DNA sequences and

vectors containing may be, and typically are, provided in solution, for example in aqueous solution containing buffers or in the usual culture media.

Substantial Sequence Homology: Substantial sequence homology means close structural relationship between sequences of nucleotides or amino acids. For example, substantially homologous DNA sequences may be 80% homologous, preferably 90% or 95% homologous, and substantially homologous amino acid sequences may typically be 50% homologous, or more. Homology also includes a relationship wherein one or several subsequences of nucleotides or amino acids are missing, or subsequences with additional nucleotides or amino acids are interdispersed.

## ***ABBREVIATIONS***

The following abbreviations are used herein:

| | |
|---|---|
| AHAS | acetohydroxyacid synthase |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| bp | base pair |
| BT | Bacillus thuringiensis endotoxin |
| BTH | methyl benzo-1,2,3-thiadiazole-7-carboxylate |
| CAT | chloramphenicol acetyltransferase |
| CETAB | hexadecyltrimethylammonium bromide |
| CHX | cycloheximide |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| DTT | dithiothreitol |
| dicamba | 3,6-dichloro-2-methoxybenzoic acid |
| EDTA | ethylendiamine N,N,N′,N′-tetraacetic acid |
| GUS | beta-l,3-glucuronidase |
| INA | isonicotinic acid |
| kb | kilo base pair |
| LUX | luciferase |

| | |
|---|---|
| MES | 2-(N-morpholino)ethanesulfonic acid |
| MU | 4-methyl umbelliferyl glucuronide |
| NOS | nopaline synthase |
| NPT | neomycin phosphotransferase |
| NRRL | designation for deposits made with the Agricultural Research Culture Collection, International Depositing Authority, 1815 N. University Street, Peoria, Illinois 61604 |
| OCS | octopine synthase |
| PEG | polyethylene glycol |
| picloram | 4-amino-3,5,6-trichloropicolinic acid |
| PR protein | Pathogenesis-related protein |
| SA | salicylic acid |
| SAR | Systemic Acquired Resistance |
| SDS | sodium dodecyl sulfate |
| TFA | trifluoroacetic acid |
| TMV | tobacco mosaic virus |
| Tris-HCl | tris(hydroxymethyl)methylamine hydrochloride |
| WCI | Wheat Chemically Induced (gene nomenclature designation) |

***ANTI-PATHOGENIC SEQUENCES***

The present invention also embraces anti-pathogenic DNA sequences which are capable of conferring enhanced disease resistance or disease tolerance when expressed in a plant or plant tissue. This includes coding sequences for plant pathogenesis-related (PR) proteins as described herein and sequences with substantial homology to these coding sequences.

Included within the scope of the present invention, in addition to the sequences exemplified specifically below and enumerated in the sequence listing, are cDNA sequences which are equivalent to the enumerated sequences which encode the given plant pathogenesis-related protein, and cDNA sequences which hybridize with the enumerated sequences and encode a polypeptide having some degree of disease-resistant activity of the given plant pathogenesis-related protein (i.e. an anti-pathogenic sequence).

Equivalent cDNA sequences are those which encode the same protein even though they contain at least one different nucleotide from the enumerated sequence. As is well known in the art, the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins, different nucleotide sequences can code for a particular amino acid. Thus, the genetic code can be depicted as follows:

| Amino Acid | Codon | Amino Acid | Codon |
|---|---|---|---|
| Phenylalanine (Phe) | TTK | Histidine (His) | CAK |
| Leucine (Leu) | XTY | Glutamine (Gln) | CAJ |
| Isoleucine (Ile) | ATM | Asparagine (Asn) | AAK |
| Methionine (Met) | ATG | Lysine (Lys) | AAJ |
| Valine (Val) | GTL | Aspartic acid (Asp) | GAK |
| Serine (Ser) | QRS | Glutamic acid (Glu) | GAJ |
| Proline (Pro) | CCL | Cysteine (Cys) | TGK |
| Threonine (Thy) | ACL | Trytophan (Trp) | TGG |
| Alanine (Ala) | GCL | Arginine (Arg) | WGZ |
| Tyrosine (Tyr) | TAK | Glycine (Gly) | GGL |
| Termination signal | TAJ | | |

Key: Each 3-letter deoxynucleotide triplet codon corresponds to a trinucleotide of mRNA, having a 5′-end on the left and a 3′-end on the right. All DNA sequences given herein are those of the strand whose sequence corresponds to the mRNA sequence, with thymine substituted for uracil. The letters stand for the purine or pyrimidine bases forming the deoxynucleotide sequence as follows:

A = adenine; G = guanine; C = cytosine; T = thymine
X = T or C if Y is A or G
X = C if Y is C or T
Y = A, G, C or T if X is C
Y = A or G if X is T
W = C or A if Z is A or G

W = C if Z is C or T
Z = A, G, C or T if W is C
Z = A or G if W is A
QR = TC if S is A, G, C or T;
QR = AG if S is T or C
J = A or G
K = T or C
L = A, T, C or G
M = A, C or T

The above shows that the amino acid sequence of the instant plant pathogenesis-related proteins can be prepared using different nucleotide sequences encoding the same amino acid sequence of the proteins. Accordingly, the scope of the present invention includes such "equivalent nucleotide sequences."

cDNA sequences that hybridize with a given enumerated sequence and encode a polypeptide or protein having at least some degree of activity of the corresponding plant pathogenesis-related protein are those which exhibit substantial sequence homology, as defined hereinabove, with the enumerated Sequence such that it hybridizes with the latter under low stringency conditions. Such conditions are described in Examples 17 and 20 of European patent application EP-A-0 392 225. Proteins translated from these hybridizable cDNA sequences have different primary structures from proteins translated from the enumerated Sequences. However, their respective secondary structures are the same. Thus, the former proteins retain some degree of the biological activity (plant disease resistance) of the latter proteins and are considered as plant pathogenesis-related proteins also, e.g. "a PR-O protein," "a PR-N protein," etc. As used elsewhere herein, the term "activity" is referred to as the property of enhanced disease resistance or tolerance conferred upon a transgenic plant by virtue of its ability to express the DNA encoding the PR protein

It is contemplated by the invention that increased disease resistance or tolerance or increased scope of protection against a broader range of pathogens can be acheived by providing for the expression of more than one anti-pathogenic DNA sequence in a transgenic plant or plant tissue. The increased disease resistance thus achieved is due to a synergistic effect created by expressing a combination of anti-pathogenic sequences in the

same plant or plant tissue. Thus, a method is provided which improves protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins excert a synergistic effect. Preferably the DNA molecules encode anti-pathogenic proteins according to the invention. Due to synergy, the anti-pathogenic effect of a particular sequence may be more readily detected when combined with one or more other anti-pathogenic sequences than when present alone.

The present invention further includes chimeric genes and vectors comprising one or more anti-pathogenic sequences analogous to the chimeric genes and vectors comprising chemically regulatable sequences described in parts F and G above. Any desired promoter may be used in association with the anti-pathogenic sequences of the invention, including promoters which confer constitutive expression, promoters which confer expression in a particular tissue or subset of tissues and/or at a particular developmental stage, and promoters which confer chemically regulatable expression containing the noncoding sequences taught herein.

Also included as part of the present invention are plant tissues, plants and seeds comprising anti-pathogenic sequences analogous to those plant tissues, plants and seeds comprising chemically regulatable sequences described in part H above.

## ***DIFFERENTIAL CLONING AND SCREENING TECHNOLOGY***

A method has been conceived and developed which will allow efficient enrichment of sequences present in one population of molecules in greater amounts than in another population. The method's greatest utility is in situations where the populations are very similar and the differentially present sequences represent a very small proportion of the population.

If two populations of clones are similar and one wishes to isolate those clones which are present in one population in higher amounts (i.e. "induced" or differentially regulated"), past techniques involved screening with probes from the two populations (+/- screening; St. John and Davis, *Cell 16:*443-452 (1979)), or enrichment of probes or mRNAs by hybridization and hydroxy-apatite (HAP) chromatography (Davis, *et al., Proc. Natl. Acad. Sci, USA 81:* 2194-2198 (1984)). The first method has a demonstrated sensitivity limitation

in that only clones present in greater than about one in 2,000 will be detected. The second is laborious, technically difficult, and achieves enrichments of 20-50 fold at best.

The present method involves exploiting two recent developments in molecular technology: the polymerase chain reaction (Saiki *et al., Science 239:*487-491 (1988)) and biotin-avidin chromatography [Stahl, *et al., Nuc. Acids. Res. 16:* 3026-3038 (1988)). The polymerase chain reaction (PCR) allows simple synthesis of large amounts of DNA of specified sequence. Biotin-avidin chromatography allows the efficient separation of molecules bearing a biotin affinity tag from those molecules which do not bear the tag.

In its general form, the technique consists of isolating single strands of cDNA representing two different populations ("induced" vs "uninduced"), but of opposite cDNA polarity for the two populations, i.e. one of "sense" polarity relative to mRNA's, and the other its complement, or "anti-sense", polarity relative to mRNA's. The isolated strands from the "induced " population would have no affinity tag, while the strands of opposite polarity from the "uninduced" populations would have stable affinity tags When these two populations are hybridized together, hybrids will form between complementary strands present in the two populations. Those strands from the "induced" population which have no counterparts, or many fewer counterparts, in the "uninduced" population, remain single stranded

Due to the presence of the affinity tag (in essence a handle) on the strands of the "uninduced" population molecules, those strands and, most importantly, any hybrid molecules can be removed from the mixture by affinity chromatography. This leaves only those "induced" molecules which are not significantly represented in the "uninduced population. These "induced" molecules can then be cloned by standard means and serve as an enriched population from which to isolate "induced" clones; alternatively, the enriched molecules can be amplified individually and sequenced directly.

An alternate scheme is the same as described above except that it involves incorporating a labile affinity tag only on the "induced" population molecules, while the affinity tag on the "uninduced" population is stable. "Labile" in this case means that the affinity tag can be removed at will, or be altered at will in such a way that it no longer serves as an affinity tag. In this scheme all the molecules in the hybridization mixture could bind to the affinity matrix, but only those "induced" molecules that are not hybridized to a complementary "uninduced" counterpart could be selectively recovered from the matrix for subsequent cloning.

The advantage of the methods of the invention described above over those previously described is the ability to isolate those genes which are turned on only to low levels, in specific circumstances, and which may play a causative role in some important biological phenomenon.

The present invention teaches the cloning of SAR genes by differential screening of tissues induced and non-induced to the systemic acquired response SAR induction causes the transcription of genes in a protein synthesis-dependent fashion and also a protein synthesis-independent fashion. Two methods were used to clone specifically genes whose induced transcription is protein-synthesis independent. Firstly, cDNAs which were cloned by standard differential screening techniques were further screened on SAR-induced RNA isolated with and without cycloheximide (CHX) pre-treatment. Secondly, a PCR-based "differential display" technique was used to identify SAR-induced, but protein synthesis independent cDNAs directly. Differential display RNAs were prepared with and without SAR induction and CHX treatment. The use of CHX as an inhibitor of protein synthesis is well known in the art and is described by Greenberg *et al., Mol. Cell. Biol. 6:* 1050-1057 (1986), Lau and Nathans, *Proc. Natl. Acad. Sci. 84:* 1182-1186 (1987), and Uknes *et al., Plant Cell 5:* 159-169 (1993). Thus, a number of genes were cloned which were induced by the SAR response, yet expressed independently of protein synthesis. These cloned genes are likely signal transducers in the pathway leading from induction to the development of the resistant state.

## *EXAMPLES*

***Example (i): Ligation in Agarose***

Following restriction digestion of plasmid DNA and electrophoretic separation of the fragments on a low melting TAE gel, the bands containing appropriate fragments are precisely excised and heated to 65°C to melt the agarose 2-5 µl are added to 15 µl water and the solution is left at 65°C for 10 minutes. This solution is cooled to 37°C and left for five minutes to equilibrate to temperature 2 µl of 10 X ligase buffer (200 mM Tris, pH 8.0, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP) are added along with 1 µl T4 DNA ligase (New England BioLabs), and this solution is allowed to solidify and incubate at 15°C overnight.

***Example (ii): Transformation From Agarose***

The agarose containing the appropriate DNA is melted by incubating at 65°C for 10 minutes 10 µl of this solution are added to 30 µl of TE buffer (10 mM Tris pH 7.5, 1 mM EDTA), mixed and allowed to stand at room temperature. Frozen competent cells (E. coli strain DH5) are placed on wet ice to thaw. The diluted DNA solution is added to 200 µl of cells and allowed to stand on ice for 20 minutes. The cells containing the DNA are then heat-shocked for 90 seconds at 41°C. The cells are then left at room temperature for 10 minutes 0.8 ml of SOC medium (Hanahan, D., *J. Mol. Biol. 166:* 557-580 (1983)) is added and the culture is incubated at 37°C for one hour 100 µl of the culture is plated on LB plates (Miller, supra) containing 100 µg/ml ampicillin (L-amp) and the plates are incubated overnight at 37°C. Positive colonies are picked and restreaked to a second L-amp plate and the plates are incubated overnight at 37°C.

***Example (iii): Labelling DNA Restriction Fragments***

DNA is treated with the appropriate restriction enzymes and fragments are separated by electrophoresis on a low-gelling temperature agarose gel. A band containing the fragment of interest is excised and the DNA purified by standard techniques 50 ng of the DNA fragment is labelled ysing the IBI Random primer kit "Prime time" according to the manufacturers directions.

***Example (iv):*** ***Southern Blotting***

3 µg of tobacco DNA is digested with various restriction enzymes under the conditions suggested by the supplier. The DNA is extracted with phenol, precipitated with ethanol and then resuspended in gel loading buffer (15% ficoll, 0.025% bromophenol blue, 10 mM EDTA, pH 8). Samples are loaded and electrophoresed on a 0.5% agarose gel at 5 V/cm until the bromophenol blue dye reaches the end of the gel. The DNA is transferred to Gene-Screen Plus (DuPont) using the alkaline transfer procedure as described by the supplier. Pre-hybridization, hybridization and washing are according to the manufacturer's recommendation. Hybridization is detected by autoradiography.

***Example (v):*** ***Molecular Adaptors***

A typical molecular adaptor for the conversion of a PstI site to a BamHI site is the sequence

5′-GGGATCCCTGCA-3′ (SEQ ID No. 22).

This molecule is synthesized on an Applied Biosystems Synthesizer using B-cyano-ethylphosphoramidite chemistry and purified by reverse-phase HPLC. About 2 µg of this oligonucleotide is kinased according to Maniatis *et al.*, supra, p. 125. The oligonucleotide solution is heated to 65°C in a water bath and allowed to cool to room temperature over a period of about 30 minutes. An approximately 10-fold molar excess of this annealed adapter is added to the digested DNA along with 10 X ligase buffer, T4 DNA ligase, and an appropriate amount of water. A typical reaction is:

| | |
|---|---|
| DNA to be adapted: | 1-2 µl (~ 1 pmol) |
| Adapter: | 1 µl (~10 pmol) |
| 10 X ligase buffer: | 1 µl |
| T4 DNA ligase: | 1 µl |
| Water: | 5-6 µl |

This solution is incubated at 12-15°C for 30 minutes, and heated to 65°C for 30 minutes to inactivate the ligase. The salt concentration and volume are adjusted for the appropriate restriction digest and the adapted DNA is digested to expose the adapted

"sticky end." Unincorporated adapters are removed either by electrophoresis on an agarose gel or by sequential isopropanol precipitations.

***Example (vi): Primer Extension Mapping***

A. Synthesis and 5′ End Labeling of Primers for Primer Extension

The following primer oligomers are synthesized using an Applied Biosystems Synthesizer and ß-cyanoethylphosphoramidite chemistry:

PR-1: 5′-ATAGTCTTGTTGAGAGTT-3′ (SEQ ID No. 23)

GUS: 5′-TCACGGGTTGGGGTTTCTAC-3′ (SEQ ID No. 24)

AHAS: 5′-AGGAGATGGTTTGGTGGA-3′ (SEQ ID No. 25)

BT: 5′-ATACGTTCTACTATCATAGT-3′ (SEQ ID No. 26)

The oligonucleotides are purified by reverse-phase high pressure liquid chromatography (HPLC) 5 pmol of each oligo is kinased (Maniatis, T. *et al.*, supra, at p. 125) using 200 µC of $^{32}$P-ATP (6000 Ci/mmol, 10 µCi/µl). After incubation at 37°C for 30 minutes, the reaction is diluted to 100 µl, extracted with phenol/chloroform and then precipitated three times with 50 µg carrier RNA. The final precipitate is resuspended in 1 X reverse-transcriptase buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 3 mM $MgCl_2$) at a concentration of 2 nM. The specific activity of the labeled oligonucleotide is determined to be about 3 X $10^6$ Cvcpm/pmol.

B. Total RNA Preparation

Total RNA is prepared essentially as described by Lagrimini, L.M. *et al., Proc. Natl. Acad. Sci. USA 84:* 7542 (1987). Tissue is ground under liquid nitrogen in a mortar and pestle. The ground tissue is added to grinding buffer (Lagrimini *et al.*, supra) using 2.5 ml per gram tissue. An equal volume of phenol is then added and the emulsion is homogenized in a Brinkman polytron. A one-half volume of chloroform is added and the emulsion is gently mixed for 15 minutes. The phases are separated by centrifugation and

the aqueous phase is removed. RNA is precipitated by the addition of sodium acetate to 0.3 M and 2.5 volumes ethanol. The precipitate is collected by centrifugation and resuspended in 2 ml sterile water. Lithium chloride is added to a final concentration of 3 M and left at 4°C overnight. The precipitate is collected by centrifugation and the pellet is washed with ice-cold 80% ethanol. The pellet is dried and resuspended in 500 µl sterile water. The concentration of this total RNA preparation is determined spectrophotometrically.

Alternatively, RNA is extracted from callus as described above except that the callus tissue is cut into cubes approximately 3 mm in size, and added to pre-chilled mortars and pestles for grinding in liquid nitrogen prior to the polytron step.

C. Primer Extension

50 µg of total RNA is lyophilized in a 500 µl Eppendorf tube. The RNA is resuspended in 30 µl of radiolabeled probe solution and heated to 70°C for 10 minutes. The tube is slowly cooled to 37°C and allowed to incubate overnight Without removing the tube from the 37°C water bath, 2 µl of 10 X reverse-transcriptase buffer (500 mM Tris-HCl, pH 7.5, 400 mM KCl, 30 mM $MgCl_2$), 1 µl 5 mg/ml bovine serum albumin, 5 µl 100 mM dithiothreitol, 5µl 10 X dNTPs (10 mM of each dNTP in $H_2O$), 3 µl $H_2O$, 2 µl RNasin (80 units), and 2 µl reverse transcriptase (400 units) are added and the reaction is incubated at 37°C for 30 minutes. To stop the reaction, 5 µl of 3 M sodium acetate, pH 5, and 150 µl absolute ethanol are added. The tube is left at -20°C for 30 minutes, the precipitate is collected by centrifugation, washed with 80% ethanol and allowed to air-dry. The precipitate is resuspended in 10-20 µl of loading dye (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 1 mM EDTA) and the extension products are separated on a 6% sequencing gel (Maniatis, T. *et al.*, supra). Extension products are visualized by autoradiography.

***Example (vii): S1 Nuclease Mapping***

The plasmid pBS-PR1013Cla is digested with SfaNI, dephosphorylated with calf intestinal phosphatase and kinased with $^{32}$P-ATP. Following phenol extraction and ethanol precipitation, the DNA is digested with BstEII and the 300 bp fragment from 750 to 1035 of Figure 1 is isolated after electrophoresis on a low gelling temperature agarose gel. The probe is resuspended in formamide hybridization buffer (Berk, A.J. *et al.*, Cell 12, 721

(1977)) at a concentration of about 2 nM. The specific activity of the probe is about 5 X 10 Cvcpm/pmol.

Lyophilized, total RNA (50 µg) is dissolved in 30 µl of the probe solution, and the tubes are heated to 65°C for 10 minutes, then allowed to hybridize overnight at 48°C. S1 nuclease treatment and gel electrophoresis are essentially as described, using an S1 concentration of 400 units/ml and an incubation temperature of 30°C. The appropriate S1 nuclease concentration is determined in pilot experiments.

***Example (viii): Mapping the Transcriptional Start Site***

The transcriptional start site for the PR-1a gene is determined by a combination of S1 nuclease mapping and primer extension analysis. An autoradiogram of a primer extension experiment using either RNA isolated from TMV-infected leaves or an mpl9 subclone of the XhoI-PstI fragment as a template and a 17 base oligonucleotide complementary to positions 1025 to 1042 of the PR-1a sequence as a specific primer is examined. The primer itself is labeled at the 5′ phosphate, therefore the size of the extension product will be identical to the size of the corresponding band in the sequencing lanes. The appearance of two strong bands corresponding to positions 902 and 903 and a weak band at position 901 of the genomic clone suggests transcription initiating at either of these positions. However, primer extension analysis alone cannot be used to identify the 5′ end of a mRNA. For instance, the mRNA may contain a 5′ end that has been spliced from an upstream location.

To determine conclusively the 5′ end, high resolution S1 nuclease mapping is used in conjunction with primer extension. An SfaNI fragment is labeled at the 5′ end and digested with BstEII to yield a strand specific probe extending from position 759 to 1040. This probe is used to map the 5′ end of PR-1a transcripts in RNA isolated from TMV-infected tobacco leaves. A major band of 137 2 bases is found which corresponds to positions 901 to 905 of the genomic clone. In high resolution S1 experiments, where the digestion products are electrophoresed along with a size standard of sequencing reactions performed on the probe, three bands are visualized corresponding to positions 901, 902 and 903. These results confirm the primer extension analysis and place the 5′ end of the PR-1 mRNA at either position 901, 902 or 903. With regard to transcription initiation, one possible interpretation of these results is that RNA polymerase begins transcription at either base 901, 902 or 903 with more or less equal probability. However, since eukaryotic

transcription favors initiation at an A, a more likely explanation for the apparent multiple 5′ ends is that the PR-1a mRNA begins at position 903 (an A) and the PR-1b and -1c mRNAs begin each at one of the other positions on their corresponding genes.

***Example (ix): General Techniques for Peptide Generation, Purification, and Automated Sequencing***

The proteins relevant to these examples are isolated, purified and sequenced in some cases, for the first time and in accordance with literature procedures in other, for the purpose of allowing the isolation of the corresponding cDNA's and ultimately for confirming the identities of their cDNA's and chemically inducible genes.

A. Reduction and Alkylation

Purified, lyophilized protein is dissolved in 6 M guanidine-HCl containing 1 M Tris-HCl, pH 8.6, 10 mM EDTA. Dithiothreitol is added to 20 mM and 4-vinylpyridine is added to a final concentration of 50 mM. The sample is then incubated for 1.5 hours under nitrogen. The pyridylethylated material is desalted on HPLC using an Aquapore phenyl column (2.1 x 10 cm, Brownlee). The column is eluted with a linear, 5-80% gradient of acetonitrile/isopropanol (1:1) in 0.1% trifluoroacetic acid (TFA).

B. Cyanogen Bromide Cleavage and Removal of Pyroglutamate

Cyanogen bromide cleavage is performed in situ according to Simpson, R.J. *et al., Biochem. Intl. 8:* 787 (1984) Digestion of PR-1 protein with pyroglutamate aminopeptidase (Boehringer Mannheim) is carried out according to Allen, G., *Plant Sci. Lett. 26:* 173 (1982).

C. LysC digestion

Protein is digested with endoproteinase Lys-C (Boehringer Mannheim) in 0.1 M Tris-HCl, pH 8.5, for 24 hours at room temperature using an enzyme:substrate ratio of 1:10. Resulting peptides are isolated by HPLC using an Aquapore C-8 column (1 x 22 cm, Brownlee) eluted with a linear acetonitrile/isopropanol (1:1 ratio) gradient (0 to 60%) in 0.1% TFA.

D. Trypsin Digestion

Digestion with trypsin (Cooper) is performed in 0.1 M ammonium bicarbonate, pH 8.2, containing 0.1 M calcium chloride for five hours at 37°C using an enzyme:substrate ratio of 1:100. Peptides generated are separated on HPLC using the same conditions as with the Lys-C peptides or performed in 0.1 M Tris-HCl pH 8.5 for 24 hours at 37°C using an enzyme to substrate ratio of 1:50. Peptides are isolated by HPLC using a Vydac C-18 column (2.1 x 150 mm) with a linear 0 to 60% acetonitrile:isopropanol (1:1) gradient in 0.1 % TFA.

E. Sequencing

Automated Edman degradations are performed with an Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin (PTH) amino acids are identified using an Applied Biosystems 120A PTH analyzer.

***Example 1A: Cloning of cDNAs corresponding to SAR CHX-independent genes from tobacco***

A number of cDNAs were cloned by differential screening from cDNA prepared from induced and non-induced tissue. The induced cDNA was prepared from tobacco leaves which had been pre-treated with methyl benzo-1,2,3-thiadiazole-7-carboxylate (BTH), whereas the non-induced cDNA was prepared from tissue which had not been pre-treated with BTH. cDNA libraries were prepared in λZAP II (STRATAGENE). A standard differential screening technique was used. Plaques carrying induced cDNA were plated at low density and transferred to two sets of hybridization filters. Known SAR gene sequences were hybridized to the first filter and uninduced cDNA to the second. The second filter was then stripped and hybridised with induced cDNA. Plaques which hybridized with the induced cDNA probe, but not with the uninduced cDNA probe or with known SAR gene sequences were potential novel SAR genes and were picked directly for further analysis. Plating had been at a plaque density which was sufficiently low to enable these plaques to be picked as nearly pure plaques. Multiple candidates for each plaque were *in vivo* excised, according to the manufacturter's recommended conditions, for further screening by Northern hybridization to RNA isolated from either untreated or BTH-treated tobacco plants. Individual clones chosen from the secondary screen were further analyzed by Northern

hybridisation to RNA isolated from tobacco plants which had been pre-treated with salicylic acid (3 mM), INA (1 mM), and BTH (1 mg/ml), all in the presence or absence of cycloheximide (CHX; 1 mg/ml). Inducer pre-treatments were done at 2 h, one day, and eight days before the isolation of RNA, whereas CHX treatment was done at one day before isolation of RNA. Three cDNAs were found induced in a protein synthesis independent fashion. The genes corresponding to these cDNAs have been designated p66B1 (SEQ ID NO. 14), p11.30.13 (SEQ ID NO. 11), and p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) and are likely signal transducers of the SAR response. Gene p1.4.3 has previously been disclosed as a thioredoxin (Brugidou *et al., Mol. Gen. Genet. 238:* 285-293 (1993)). However, this is the first disclosure of the gene's likely involvement in the systemic acquired response. Three cDNAs were found to be expressed in a protein synthesis dependent fashion. These were designated p1.1.1, p11.31.4, and p14.22.3 and are listed as SEQ. ID Nos. 9, 10, and 15, respectively.

***Example 1B: Cloning of cDNAs corresponding to SAR CHX-independent genes from Arabidopsis using INA for induction***

Total RNA was isolated from the following Arabidopsis lines: (1) untreated, (2) INA treated (0.25 mg/ml), (3) CHX treated (1 mg/ml), and (4) INA + CHX treated. Treatments were made 1 day before RNA isolation. RNA thus isolated was subjected to "differential display" using the protocol described by Liang and Pardee, *Science 257:* 967-971 (1992). Amplified fragments which were found in both the INA as well as the INA + CHX treated RNA samples were gel-purified and used as probes on Northern blots carrying similarly induced RNA samples. Fragments for which Northern hybridization confirmed the induction profile apparent from differential display were subcloned into a plasmid vector. Using the cloned fragment it was possible to isolate near full-length cDNAs from a cDNA library produced by BTH induction:

Mature Arabidopsis thaliana ecotype Columbia (Lehle Seeds, Tucson, AZ) plants are sprayed with a 0.5 mg/ml suspension of a wettable powder formulation of methylbenzo-1,2,3-thiadiazole-7-carboxylate consisting of 25% active ingredient. Seven days later, the leaf tissue is harvested and frozen in liquid $N_2$. Total RNA is isolated as described in Example (vi) and Poly $(A)^+$ RNA is isolated using a Poly (A) Quik™ mRNA isolation kit from Stratagene (La Jolla, CA). This Poly $(A)^+$ RNA is then used to make a cDNA library in the

uni-zap$^{TM}$ XR vector (Stratagene) using a ZAP-cDNA$^{TM}$ Gigapack$^{R}$ II Gold cloning kit from Stratagene. A portion of the cDNA library is amplified as described in the Stratagene kit.

The cDNA pDPA2 was cloned using this technique and is induced by the SAR response in a protein synthesis independent fashion. Its sequence is listed in Seq. ID No. 16.

***Example 1C: Cloning of cDNAs corresponding to SAR CHX-independent genes from Arabidopsis using BTH for induction***

RNA isolated from (1) untreated, (2) BTH treated, and (3) BTH + CHX treated Arabidopsis lines was subjected to "differential display" using the protocol described by Liang and Pardee, *Science 257:* 967-971 (1992) and as described in example 1B above. Amplified fragments which were found in both the BTH as well as the BTH + CHX treated RNA samples were gel-purified and used as probes on Northern blots carrying similarly induced RNA samples. Fragments for which Northern hybridization confirmed the induction profile apparent from differential display were subcloned into a plasmid vector. The following clones induced in a protein synthesis-independent manner were obtained:

PSI-1 (SEQ ID NO. 33): cDNA homologous to EMBL Accession #Z26429, an A. thaliana transcribed sequence (clone FAFC80-1; 5' end and actin depolymerizing factor from L. longiflorum), and EMBL Accession #Z14110, an L. longiflorum mRNA for actin depolymerizing factor

PSI-2 (SEQ ID NO. 34): cDNA with no known homology

PSI-3 (SEQ ID NO. 35): cDNA homologous to EMBL Accession # T20772, A. thaliana cDNA 92H8T7, and EMBL Accession #T04101, A. thaliana cDNA clone SCG7T7P

PSI-4 (SEQ ID NO. 36): cDNA homologous to EMBL Accession #D22118, a partial Rice cDNA sequence

PSI-5 (SEQ ID NO. 37): cDNA with no known homology

***Example 2A: Cloning of Class IV Chitinase cDNAs***

To clone recombinant or chimaeric DNA molecules encoding class IV chitinase class specific degenerate oligonucleotides were designed from areas of homology between bean PR4 chitinase (Margis-Pinheiro *et al., Plant Mol. Biol. 17:* 243-253 (1991)) and sugar beet chitinase 4 (Mikkelsen *et al.,* in "Advances in Chitin and Chitosan, ed. by Brine *et al.,* pub. by Elsevier, Amsterdam (1992)): oligonucleotides were designed degenerate for the peptide

sequences HFCYIEE (forward, spanning nucleotides 406-426; SEQ ID NO. 27), and IRAING (reverse, spanning nucleotides 705 to 675; SEQ ID NO. 28). DNA was extracted from two-week old plants of Arabidopsis thaliana ecotype Landsberg and amplified using a Perkin-Elmer thermal cycler 480 at the following cycle settings: 94°C for 5 minutes; 35 cycles at 94°C, 1 minute, 43°C or 45°C, 1 minute, and 72°C, 2 minutes; followed by 5 minutes at 72°C. The amplified fragment was gel purified, collected by centrifugation through Whatman paper, ethanol precipitated, resuspended in TE, digested with BamHI and NsiI and subcloned into pTZ18U (Pharmacia). Four clones of the fragment were sequenced; they differed only within the oligo-derived sequence as could be expected from amplification with degenerate oligonucleotides.

Two different cDNAs were isolated simultaneously by screening a leaf tissue cDNA library (Uknes *et al., Plant Cell 4:* 645-656 (1992)) at high stringency with the PCR amplified genomic fragment described above. Duplicate plaque lifts were taken with nitrocellulose filters (Schleicher & Schuell, Keene, NH) (Ausubel *et al.*, in "Current Protocols in Molecular Biology, pub. by J. Wiley & Sons, New York (1987)). Probes were labelled by random priming (using the labeling system supplied by Gibco BRL, Gaithersburg, MD). Hybridization and washing were done at 65°C according to Church and Gilbert, *Proc. Natl. Acad. Sci. 81:* 1991-1995 (1984). Positive plaques were purified and plasmids containing the cDNA inserts were *in vivo* excised for DNA sequence determination. Of the six positive clones two contained an insert with structural homology to previously characterized class IV chitinases and were designated class IV chitinase type A and four clones were divergent in that they lacked the class IV chitinase hevein domain; these were designated class IV chitinase type B. As none of the cDNA inserts was full-length, an additional 29 bp of class IV chitinase type A and 17 bp of class IV chitinase type B, both containing a methionine initiation codon were amplified from ethephon-induced RNA using the 5' RACE system for rapid amplification of cDNA ends (Gibco BRL, Gaithersburg, MD). Sequence comparisons were performed using the GAP and PILEUP features of the Genetics Computer Group software (Genetics Computer Group, Madison, WI).

Class IV chitinase type A is a 1079 base pair cDNA with an open reading frame of 264 amino acids containing the characteristic cysteine-rich hevein and chitinolytic domains and the three short deletions typically found in class IV chitinases (see SEQ ID No. 1). The cDNA for class IV chitinase type B is 952 base pairs in length and encodes a protein of 214 amino acids which lacks a hevein domain and contains a fourth deletion (see SEQ ID No.

2). The cDNAs are 71% identical overall and 80% identical over coding sequence. The predicted protein encoded by Arabidopsis class IV chitinase type A is 89% homologous to the basic Brassica napus (rapeseed) class IV chitinase, 61% homologous to basic Beta vulgaris (sugar beet) class IV chitinase, 57% homologous to basic Zea mays class IV chitinase B, 58% homologous to acidic Phaseolis vulgaris (bean) PR4 class IV chitinase, and 55% homologous to acidic Dioscorea japonica (yam) class IV chitinase. It is 42% homologous to Arabidopsis thaliana basic class I chitinase.

The predicted mature protein encoded by Arabidopsis class IV chitinase type A has a molecular weight of 25695 D and a pI of 7.8; whereas the protein encoded by Arabidopsis class IV chitinase type B has a molecular weight of 20553 D and a pI of 10 assuming the removal of a signal peptide based on homology to tobacco PR-Q.

Northern analysis showed that both chitinases were induced by TCV infection, confirming their classification as PR-proteins.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 2B: Isolation of Chemically Induced Wheat cDNAs Using the Method of Differential Plaque Filter Hybridization***

Recombinant or chimaeric DNA molecules comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a plant were obtained in the following way:

Samples of winter wheat (cultivar Kanzler) were harvested 2-3 days after treatment with either water or 200 ppm of the plant activator compound benzo-1,2,3-thiodiazole-carboxylic acid. Total RNA was prepared from frozen tissue samples using a standard phenol extraction/LiCl precipition procedure (Lagrimini *et al., Proc. Natl. Acad. Sci. 84:* 7542-7546 (1987)). PolyA (+) RNA was purified from total RNA using the Poly(A) Quik mRNA purification kit (Stratagene Cloning Systems, LaJolla, CA). A bacteriophage lambda ZAP II cDNA library was prepared from the benzo-1,2,3-thio-diazolecarboxylic acid treated polyA(+) sample using the Uni-Zap XR Gigapack II Gold cloning kit (Stratagene) as described by the manufacturer. The phage library was plated at a density of approximately 5000 plaques on a 10 cm petri dish and grown for 6-8 hours at 37°C. Duplicate filter lifts of

the plaques were made using nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Labelled first strand cDNA probes were prepared from polyA of both the water control and the benzo-1,2,3-thiodiazolecarboxylic acid-treated samples using $^{32}$P-dCTP and the AMV reverse transcriptase (GibcoBRL, Gaithersburg, MD) under the manufacturer's conditions. Each probe was hybridized ( >106 cpm/ml) with one set of the duplicate lifts overnight at 65°C. Hybridization and wash conditions were as described in Church and Gilbert, *Proc. Natl. Acad. Sci. 81:* 1991-1995 (1984). Hybridization was detected by autoradiography.

Plaques appearing to hybridize preferentially to the chemically treated cDNA were purified and their cDNA inserts were amplified using the GeneAmp Polymerase Chain Reaction (PCR) kit (Perkin Elmer, Norwalk, CT) and primers homologous to the flanking lambda Zap II sequences. The amplified inserts were excised from a low melting temperature SeaPlaque GTG agarose gel (FMC BioProducts, Rockland, ME) and labelled using $^{32}$P-dCTP and the Random Primers DNA Labeling System (Gibco BRL). These probes were hybridized with total RNA blots (Ausubel *et al.*, in "Current Protocols in Molecular Biology, pub. by J. Wiley & Sons, New York (1987)) of control and benzo-1,2,3-thiodiazolecarboxylic acid treated RNAs to verify that they contained chemically induced cDNAs.

The induced clones were *in vivo* excised into pBluescript plasmids according to the manufacturer's instructions (Stratagene) and plasmid DNAs were purified using Magic Miniprep columns (Promega Biotech, Madison, WI). The cDNA sequences were determined by the chain termination method using dideoxy terminators labelled with fluorescent dyes (Applied Biosystems, Inc., Goster City, CA). The DNA and the predicted amino acid sequences were compared to available databases using the GAP (Deveraux *et al., Nucl. Acids Res. 12:* 387-395 (1984)) and the BLAST (Altschul *et al., J. Mol. Biol. 215:* 403-410 (1990)) programs.

The nucleotide sequence of one apparently full length induced clone is set forth in SEQ ID No. 3. This clone, denoted WCI-1 (Wheat Chemical Induction), was found to share limited homology with two rice cDNAs, one expressed specifically in the shoot apical meristem (De Pater and Schilperoort, *Plant Mol. Biol. 18:* 161-164 (1992)), the other inducible by salt stress (Claes *et al., Plant Cell 2:* 19-27 (1990)). The function of these proteins is unknown.

The predicted amino acid sequence of a second highly induced cDNA, WCI-2, clearly identified it as an isozyme of wheat lipoxygenase, based on its homology to other

plant lipoxygenases in the database. The DNA sequence of this cDNA is shown in SEQ ID No. 4.

A third class of induced cDNA (WCI-3) was isolated which to date shows no significant homology to sequences in the databases. The DNA sequence of this apparently full-length clone is set forth in SEQ ID No. 6.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 2C: Isolation of Wheat cDNAs Specifically Induced by Treatment With benzo-1,2,3-thiodiazolecarboxylic acid, Using the Method of Differential cDNA Display***

The total and polyA(+) samples described in Example (vi) were used for PCR differential display of the mRNA from water and benzo-1,2,3-thiodiazolecarboxylic acid treated wheat tissue essentially as described in Liang and Pardee, *Science 257:* 967-971 (1992). Amplified cDNA fragments that appeared to be present only in the chemically treated sample were excised from the dried sequencing gel and electroeluted using a Centrilutor device and Centricon-30 Microconcentrators (Amicon, Beverly, MA). The purified fragments were PCR amplified using primers that consisted of the original differential display 10-mers plus an additional 10 bases of unique sequence added to their 5' ends. After 8 PCR cycles at low annealing temperature (42-45°C), the annealing temperature was raised to 60°C for an additional 30 cycles. Essentially 100% of the fragments could then be visualized by EtBr staining and were excised from a SeaPlaque GTG agarose gel (FMC).

These gel fragments were labelled and used to probe RNA blots as previously described. Fragments that hybridized only with chemically treated RNA were TA-cloned into the plasmid vector pCR II using a TA Cloning Kit (Invitrogen Corporation, San Diego, CA). Inserts from the plasmids were screened again against control/chemical RNA blots to verify that the inducible gene fragment had been subcloned. Induced fragments were then labelled by random priming and hybridized against filter lifts of the chemically induced cDNA library as described in Example 2A. Hybridizing plaques were purified, sequenced, and analyzed as previously described in order to obtain full length clones corresponding to the

original small (200-400 bp) fragments, and to identify the induced gene product where possible.

The nucleotide sequence of one clone obtained by this procedure is set forth in SEQ ID No. 7. This clone, WCI-4, has some homology to known thiol protease sequences from a variety of sources and may therefore be a thiol protease.

Partial sequences of an additional induced gene that was isolated using differential display is set forth in SEQ ID No. 8. This fragment shows no database homology and has been designated WCI-5.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 3A: Preparation of cDNA Library From TMV-Infected Tobacco Leaves.***

Nicotiana tabacum cv. Xanthi-nc leaves are infected with tobacco mosaic virus and harvested five days post-infection. Total RNA is prepared as described above and poly A+ RNA is isolated using standard techniques. A cDNA library is constructed using this poly A+ RNA in the Lambda ongC cloning vector (Stratagene) essentially as described (Gubler, U. and Hoffman, B. J., *Gene 25:* 263 (1983)).

***Example 3B: Isolation of cDNA Clones Encoding Chitinase/Lysozymes***

To clone recombinant or chimaeric DNA molecules encoding chitinase/lysozyme about 300,000 plaques of the TMV-infected tobacco cDNA library described in Example 3A are screened using a labeled cDNA probe encoding the cucumber chitinase/lysozyme cDNA and washing filters at 50°C in 0.125 mM NaCl, 1% SDS, 40 mM sodium phosphate (pH 7.2), 1 mM EDTA. Positive plaques are purified and the DNA sequence of two clones, named pBSCL2 and pBSTCL226 are determined. These are presented in SEQ ID Nos. 20 and 21, respectively. The proteins encoded in the clones of these sequences are determined to be chitinase/lysozymes based on structural homology to the cucumber chitinase/lysozyme.

In addition, a protein is purified from intercellular fluid of TMV-infected tobacco. Peptides are generated and sequenced as described in Example (ix)

The protein encoded by pBSTCL226, corresponding to an acidic isoform of chitinase/lysozyme, was found to match the deduced peptide sequences exactly.

Using techniques well known in the art, these cDNAs can be cloned into expression cassettes and vectors for transfer to transgenic plants. Typical techniques used in the art are described in section 4A (examples 22 to 35), section 5A (examples 36 to 56) and section 6 (examples 57 to 80) of EP-A 0 392 225.

***Example 3C: Analysis of Seed Lines Derived From Transformation of Tobacco with the Basic and Acidic Class III Tobacco Chitinase Genes***

Binary constructions carrying the tobacco basic and acidic class III chitinases were transformed into *Nicotiana tabacum* cv *Xanthi-nc* Leaf tissue samples were taken from T1 plants and assayed for expression of either transgene protein (by Western analysis) or transgene RNA (by Northern analysis) T1 plants found to express the transgene at high levels were advanced to T3 seed lines as described above.

***Example 3D:*** **Analysis of Transgenic Plants Expressing Class III Chitinase for Pest Resistance**

Transgenic plant lines expressing one or more class III chitinase genes are assessed for resistance to numerous pests. Approximately six plants of each line are tested. Pests at the appropriate stage of their growth cycle (such as larvae) are introduced at the appropriate stage of plant development. Plants are later assayed for % of leaf or tissue area eaten, % of introduced larvae surviving, and the weight of surviving larvae.

***Example 3E:*** **Analysis of Transgenic Tobacco Plants Expressing Class III Chitinase for Pest Resistance**

Transgenic tobacco plants expressing class III chitinase are assessed for resistance to numerous pests. Those tested include *Spodoptera exigua* (beet armyworm), the green peach tobacco aphid, *Manduca sexta*, and various nematodes, weevils, mites, and other pests.

*Example 3F:* **Analysis of Transgenic Plants Expressing Basic Class III Chitinase for Resistance to *Heliothis virescens***

Transgenic tobacco lines expressing the tobacco basic class III chitinase gene were assessed for resistance to the insect *Heliothis virescens*. Eight plants of each line were tested 50 mm leaf discs were cut from the youngest leaves of transgenic plants approximately six weeks after germination and 3 larvae (2nd stage) were allowed to feed on each disc for 3-7 days. After 3-7 days the leaf discs were assessed for area eaten, % of larval survivors and weight (in mg) of larval survivors. Results of three separate experiments are shown in the tables provided below.

The results show elevated resistance to *Heliothis* in basic class III chitinase overexpressing plants when compared to non-transformed control lines *Heliothis virescens* (the tobacco budworm) causes considerable damage in tobacco crops and is particularly recalcitrant to control using pesticides as the larvae burrow deep within the plant. Furthermore, other *Heliothis* species cause similar damage to cotton and other crops.

| (1) Line | %LEAF EATEN | %SURV IVORS | WT (mg) LARVAE | (2) | %LEAF EATEN | %SURV IVORS | WT(mg) LARVAE |
|---|---|---|---|---|---|---|---|
| Control | 72 | 100 | 23 | | 77 | 100 | 26 |
| 3505 C-15-5 | 45 | 92 | 14 | | 65 | 96 | 19 |
| 3505 C-5-3 | 55 | 96 | 17 | | 60 | 92 | 23 |
| 3505 C-4-2 | 57 | 92 | 19 | | 60 | 100 | 20 |
| 3505 C-12-8 | - | - | - | | 44 | 87 | 15 |

| (3) Line | %LEAF EATEN | %SURV IVORS | WT (mg) LARVAE |
|---|---|---|---|
| Control | 65 | 100 | 53 |
| 3505 C-12-8 | 59 | 100 | 34 |

A summary of the two experiments detailed above is given below:

| (1) Line: | Control | 3505 C-15-5 | 3505 C-5-3 | 3505 C-4-2 |
|---|---|---|---|---|
| Resistance Index | 0 | 4 | 2.5 | 2 |

| (2) Line: | Control | 3505 C-15-5 | 3505 C-5-3 | 3505 C-4-2 | 3505 C-12-8 |
|---|---|---|---|---|---|
| Resistance Index | 0 | 2 | 3 | 3 | 4 |

| (3) Line: | Control | 3505 C-12-8 |
|---|---|---|
| Resistance Index | 0 | 3 |

Resistance Index:

S = significantly more susceptible than control plants (i.e. wild-type)

(S) = more susceptible than control plants, but not significantly in all evaluations

0 = like wild type plants

1 = some signs of resistance but not significantly different to controls

2 = identified resistance, significant only in some evaluations

3 = resistance with small significant differences in all evaluations

4 = high resistance, but symptoms or damage can still be observed

5 = total resistance. No symptoms or damage.

***Example 4A: Isolation of a Genomic Clone for Arabidopsis PR-1***

The *Arabidopsis* PR-1 cDNA cloned in pAPR1C-1 (sequence SEQ ID No. 17) was used as a hybridization probe in screening an *Arabidopsis* λEMBL 3 genomic library (purchased from Clontech). Four hybridizing plaques were plaque purified using conventional techniques and λDNA was isolated from each one with lambdasorb (Promega). The λDNA thus isolated was digested with XhoI, electrophoresed, transferred to hybridization membrane for hybridization with the PR-1 cDNA. A fragment of 7 kb was found to hybridize to the cDNA. DNA from one of the four purified plaques was then redigested with XhoI and ligated into the XhoI site of pBluescript (Stratagene). A colony carrying a recombinant or chimaeric DNA molecule comprising the promoter region of the Arabidopsis PR-1 gene was identified by probing with oligonucleotide DC21 from the PR-1 coding sequence (position +110 to +84) and the plasmid contained therein was designated pAtPR1-P and deposited January 5, 1994 with the the Agricultural Researech Culture Collection, International Depositing Authority, 1815 N. University Street, Peoria, Illinois 61604 under the accession no. NRRL B-21169. Restriction analysis identified the 7 kb XhoI fragment as extending 4.2 kb upstream of the ATG of the PR1 gene.

***Example 4B: Fusion of the Arabidopsis PR-1 Promoter to the Firefly Luciferase Gene***

Plasmid pDO432 containing a gene encoding luciferase (LUC) from firefly was received from Dr David Ow (University of California, San Diego; see Ow *et al.*, 1986; Science 234: 856). The LUC gene was excised from pDO432 by digestion with XbaI (at position +45 relative to the ATG) and SstI (approximately 1.8 kb downstream of the ATG and outside the LUC coding region). Additionally, an EcoRI-XbaI promoter fragment was

excised from pAtPR1-P; this fragment was 1.4 kb in size and delineated by an XbaI site 2.8 kb upstream of the PR-1 ATG and an EcoRI site in the pBluescript polylinker distal to the 5' end of the cloned promoter fragment (at -4.2 kb relative to the ATG). These two fragments were cloned by threeway ligation into EcoRI/SacI cleaved pBluescript thus orienting the LUC gene adjacent to the upstream PR-1 promoter fragment (pAtPR1-Q).

Subsequently, pAtPR1-P was cleaved with XbaI (at the -2.8 kb position and within the pBluescript polylinker) and religated to generate a PR-1 genomic construct without the upstream 1.4 kb promoter fragment and which thus ended 2.8 kb upstream of the PR-1 ATG (pAtPR1-R). This plasmid was used as a template in PCR with a left-to-right "topstrand" primer extending from positions -237 to -214 (DC39) upstream of the PR-1 ATG (oligo A) and a right-to-left "bottomstrand" primer comprising 15 bp of LUC coding sequence extending up to the LUC ATG and a further 19 bp of PR-1 sequence extending from the ATG into the PR-1 untranslated leader (oligo B: sequence: TTT GGC GTC TTC **CAT** TTT TCT AAG TTG ATA ATG G; SEQ ID No. 18). This PCR reaction was undertaken for five cycles at 94°C (30 s), 40°C (60 s), and 72°C (30 s) followed by 25 cycles at 94°C (30 s), 55°C (60 s) and 72°C (30 s) and this generated a product of 245 bp through annealing of the homologous PR-1 sequences; the fragment included a BglII site at its left end from the PR-1 promoter. A second PCR reaction was done using plasmid pDO432 as a template and using a left-to-right "topstrand" oligonucleotide which comprised 15 bp of PR-1 untranslated leader up to the PR-1 ATG and a further 12 bp of LUC sequence from the ATG into the LUC coding sequence (oligo C: sequence: TAT CAA CTT AGA AAA **ATG** GAA GAC GCC AAA; SEQ ID No 19) and a right-to-left "bottom strand" oligonucleotide extending from positions 332 to 312 (DC53) into the LUC coding sequence (oligo D). This PCR reaction was done under the same conditions as the one described above and generated a fragment of approximately 300 bp through annealing of the homologous LUC sequences; this fragment included a PstI site at its right end, derived from the LUC sequence amplified.

The two PCR fragments generated above were gel purified using standard procedures to remove oligonucleotides and were then themselves mixed in a further PCR reaction ("inside-outside PCR") with oligonucleotides A and D as primers. Conditions for this reaction were the same as described above. The amplified fragment was a fusion of the PR-1 promoter fragment from the first PCR reaction described above and the LUC 5' coding sequence from the second PCR reaction described above and had a BglII site at its left end and a PstI site at its right end. The fragment was gel purified and cleaved with BglII and PstI

to yield a product of 545 bp in size which was cloned into pAtPR1-R which had previously been cleaved with the same enzymes. Cleavage of the resultant plasmid (pAtPR1-S) with XbaI released a PR-1 promoter fragment extending from -2.8 kb to the XbaI site downstream of the LUC ATG, the fusion point between the PR-1 promoter and the LUC coding sequence being at the ATG. This fragment was cloned into XbaI cleaved pAtPR1-Q regenerating the full-length PR-1 promoter (4.2 kb) in operational fusion to LUC (pAtPR1-R).

***Example 4C:*** **Transfer of the *Arabidopsis* PR-1 Promoter - Firefly Luciferase Gene Fusion to pCIB200**

TJS75Kan is first created by digestion of pTJS75 (Schmidhauser and Helinski, *J. Bacteriol. 164:* 446-455 (1985)) with NarI to excise the tetracycline gene, followed by insertion of an AccI fragment from pUC4K (Messing, J. and Vierra, J., *Gene 19:* 259-268 (1982)) carrying a NptI gene pCIB 200 is then made by ligating XhoI linkers to the EcoRV fragment of pCIB7 (containing the left and right T-DNA borders, a plant selectable nos/nptII chimeric gene and the pUC polylinker, Rothstein, S.J. *et al., Gene 53:* 153-161 (1987)) and cloning XhoI digested fragment into SalI digested TJS75Kan.

pAtPR1-R was cleaved with XhoI and SacI and transferred to SalI/SacI cleaved pCIB200 to create a binary vector construction (pAtPR1-S) suitable for *Arabidopsis* transformation pAtPR1-S was then transferred to *Agrobacterium tumefaciens* strain A136/pCIB542 for transfer to *Arabidopsis* Dijon-0 using the method described by Wen-jun and Forde, *Nucl. Acids Res. 17:* 8385-8386 (1989) T2 or T3 lines carrying the PR1-LUC transgene in the homozygous state were generated for chemical induction analysis.

***Example 4D: Chemical Induction of the Arabidopsis PR-1 Promoter***

Leaves of transgenic *Arabidopsis* lines carrying the PR-1 promoter-LUC gene fusion were treated by spraying with 0.15 µg/ml INA (isonicotinic acid) for 48 h. Five days later, sprayed leaves were analyzed for luciferase activity using the Promega Luciferase Assay System (Cat. # E 1500). Transgenic lines showed 144-fold induction over controls. In other assays, luciferase activity was measured by imaging lines for bioluminescence using intensified cameras (VIM) and photon-counting image processors (ARGUS-50 or ARGUS-100) from Hamamatsu Photonic Systems (Bridgewater, NJ). Leaves were sprayed with INA and subsequently with a 5 mM solution of D-luciferin (Analytical Bioluminescence

Laboratories, San Diego, CA) 24 h before and then immediately before imaging. Transgenic plants carrying the PR1-LUC fusion had strongly induced bioluminescence when compared to water-treated controls.

***Example 5:*** **Isolation of the Maize PR-1 and Thaumatin-like cDNAs**

The maize PR-1-like cDNA PR-1mz was isolated by screening a BTH-induced cDNA library of maize with a probe matching to the PR-1 barley clone HVPR1BR (EMBL data base Accession # X74940). The DNA sequence of the gene and the deduced amino acid sequence are shown in SEQ ID NOs. 29 and 30, respectively.
The maize thaumatin-like cDNA PR-5mz was isolated by screening a BTH-induced cDNA library of maize with a probe matching to a known rice thaumatin clone (Reimann and Dudler, Plant Physiol. 101:1113-1114). The DNA sequence of the gene and the deduced amino acid sequence are shown in SEQ ID NOs. 31 and 32, respectively.

***Example 6A:*** **Transformation of Disease Resistance Genes into Maize**

Ciba maize inbred line CGA00526 was transformed (Koziel, et al., Biotechnology 11:194-200, 1993) with tobacco *PR-1a*, tobacco *SAR 8.2*, and wheat *Thionin* disease resistance genes. Constructs used for transformation include the chimeric plasmid pUBA (Toki et al; Plant Physiol. 100: 1503-1507, 1992) containing the promoter, first exon, and first intron of the maize ubiquitin gene (Ubi-1), and the coding sequence of the *bar* gene conferring resistance to phosphinothricin (Thompson et al., EMBO J. 6: 2519-2523, 1987). For cointegration purposes, the coding sequence of either the tobacco *PR1a* gene (Alexander et al, Proc. Natl. Acad. Sci. USA 90: 7327-7331, 1993), the tobacco *SAR8.2* gene (Alexander et al.,1992, Mol. Plant-Microbe Inter. 5:513-515), or the thionin gene from wheat (Rodriguez-Palenzuela et al.,1988, Gene 70:271-281), each under the control of said ubiquitin promoter were constructed into this plasmid resulting in three separate constructs for transformation. PCR techniques were used to obtain suitable restriction sites around the coding sequences of *PR1a*, *SAR8.2*, and wheat thionin, respectively. Maize tissue was bombarded with each plasmid seperately or cotransformed with the *PR-1a* and *SAR8.2* constructs. Expression of the tobacco disease resistance genes *PR-1a* and *SAR 8.2*, a combination of *PR-1a* and *SAR 8.2*, and the wheat *Thionin* gene (Rodriguez-Palenzuela et al., Gene. 70:271-281, 1988) was confirmed by Northern blots.

***Example 6B:*** **Transformation of Disease Resistance Genes into Wheat**

Immature embryos of spring wheat were isolated aseptically approximately 2 weeks after pollination, and plated scutellum side uppermost on MS based medium (Murashige and Skoog, 1962 Physiol. Plant 15:473-439) supplemented with 2,4-D, glutamine and asparagine. Four to six hours prior to transformation embryos were transferred to medium also containing 0.25M mannitol. Constructs used for transformation included the chimeric plasmid pUBA (Toki et al, Plant Physiol. 100:1503-1506, 1992) containing the promoter, first exon and first intron of the maize ubiquitin gene (Ubi-1), and the coding sequence for the bar gene conferring resistance to phosphinothricin (Thomson et al, EMBO J. 6:2519-2523, 1987). This construct was co-transformed with a single plasmid construct containing the coding sequences for the two disease resistance genes PR1a (Alexander et al, Proc. Natl. Acad. Sci. USA 90:7327-7331, 1993) and the SAR 8.2 (Alexander et al, Mol. Plant-Microbe Inter. 6:513-515, 1992) from tobacco, expression of both sequences being driven by the double 35S promoter. For cloning purposes PCR techniques were used to obtain suitable restriction sites around the coding sequences of PR1a and SAR 8.2, respectively,. Plasmid DNA was precipitated onto gold particles as described in the Dupont Biolistic manual. Embryos were bombarded using the PDS 1000 biolistic device (Weeks et al, Plant Physiol 102:1977-1084, 1993) and transferred the following day to the original medium without added mannitol. Calli which developed from the bombarded embryos were selected for a morphogenic response and regenerated on medium containing Basta. Presence of transgene fragments were confirmed by PCR. Total RNA was extracted (Lagrimini et al, 1987 Plant Physiol 84:438-442) and gene expression confirmed by Northern analysis. Transformed plants contained and expressed the bar gene and the PR1a gene. Some events also expressed the SAR 8.2 gene. Plants were selfed and Basta resistant progeny obtained.

***Example 6C: Alternative Methods for Introducing and Expressing More Than One Anti-Pathogenic Sequence In Plant Tissue***

In addition to the possibility of expressing more than one transgene in transgenic lines by the sexual crossing of lines which are transgenic for one gene, the skilled artisan recognizes that an equivalent way of generating lines transgenic for more than one gene is by the use of transformation plasmids which carry more than one gene. For example, the expression of two cDNAs in a transgenic line can be achieved by the transformation of the host plant with a vector carrying each cDNA under the independent regulation of two promoters *i.e.* the vector carries two expression cassettes in addition to sequences needed for antibiotic selection *in vitro*. Each expression cassette can also carry any signal

sequence, vacuolar targetting sequence and transciptional terminator so desired. Vectors carrying multiple expression cassettes can be constructed for use with *Agrobacterium* transformation or direct gene transfer transformation systems.

A further method for the expression of more than one transgene in a transgenic plant line is to firstly transform with a single gene (with appropriate regulatory signals) carried on a transformation vector and subsequently transform a line selected from this transformation with a further gene (with appropriate regulatory signals) carried on a different plasmid which utilizes a different antibiotic selection system. This method is obvious to those of skill in the art.

***Example 6D: Synergistic Effect of Combined Anti-Pathogenic Sequences***

Overexpression of two or more PR proteins in a transgenic plant gives rise to a synergistic anti-pathogen effect. The table below shows data from an experiment in which control tobacco lines and tobacco lines rendered transgenic for PR-1a, SAR8.2, or PR-1a and SAR 8.2 were inoculated with the pathogen *Peronospora tabacina* 5 and 8 days after inoculation, percentage leaf area infected was assessed. The constructs used to obtain the transgenic tobacco lines are similar to the constructs described in examples 6A and 6B. Tobacco lines transgenic for both PR-1a and SAR8.2 were obtained according to example 6C. The left side of the table shows raw data from numerous lines of each phenotype, whereas the right side shows mean data for each phenotype. In addition to the mean values for percentage leaf area infected, the relative area compared as a percentage of the control (=100%) is presented. This enables a calculation of the expected value in the line expressing both PR-1a and SAR8.2 assuming that the individual components are additive in action. The observed values of 40.8 and 30.4 % are well below the expected values of 63.4 % (0.756 x 0.838 x 100) and 62.4 % (0.694 x 0.899 x 100) for 5 and 8 days post-inoculation. These results demonstrate that the disease resistance effects of PR-1a and SAR8.2 are synergistic in transgenic plants.

Table A

| | DAYS AFTER INOCULATION | | | MEAN DATA DAYS AFTER INOCULATION | |
|---|---|---|---|---|---|
| | 5 | 8 | | 5 | 8 |
| Control (L6) | 36.1 | 42.2 | **CONTROL** | 40.2 | 49.4 |
| Control (L18) | 42.8 | 51.1 | | (100) | (100) |
| Control (L22) | 41.7 | 55.0 | | | |
| PR-1a (L2) | 35.0 | 42.8 | **PR-1a** | 30.4 | 34.3 |
| PR-1a (L7) | 25.0 | 25.0 | | (75.6) | (69.4) |
| PR-1a (L13) | 31.1 | 35.0 | | | |
| SAR8.2 (L1) | 36.7 | 49.4 | **SAR8.2** | 33.7 | 44.7 |
| SAR8.2 (L8) | 36.1 | 45.0 | | (83.8) | (89.9) |
| SAR8.2 (L11) | 26.7 | 45.0 | | | |
| SAR8.2 (L21) | 35.6 | 46.1 | | | |
| SAR8.2 (L25) | 33.3 | 37.8 | | | |
| PR-1a/SAR8.2 (L17) | 16.1 | 15.0 | **PR-1a/SAR8.2** | 16.1 | 15.0 |
| | | | | **(40.8)** | **(30.4)** |
| Expected Additive Values: | | | | 63.4 | 62.4 |

***Example 7: Expression of SAR/CHX-independent genes in transgenic plants***

The cDNAs described in examples 1A and 1B can be expressed in transgenic plants using techniques well known in the art. As components of the signal transduction pathway involved in SAR, the CHX-independent genes are useful for the manipulation of the SAR response. For example, the constitutive expression of key components in the SAR transduction pathway in transgenic plants will likely lead to the generation of plants with

enhanced disease resistance characteristics and this will likely be achieved by the activation of components in the pathway downstream to the component being expressed transgenically and hence to the activation of anti-pathogenic end products. By way of illustration this may be achieved from the expression of the appropriate genes behind the constitutive 35S promoter cDNAs may be transferred to the vector pCGN1761 or pCGN1761/ENX which carry the double 35S CaMV promoter and the *tml* transcriptional terminator on a pUC-derived plasmid. Colonies carrying the cDNA in sense are recovered and the cDNA carrying expression cassette is subsequently excised and cloned into pCIB200 for use in plant transformations using *Agrobacterium*. For direct gene transfer, the cDNA-carrying expression cassette is transferred to the vector pCIB3064. Transformation to transgenic plants is undertaken using techniques well known in the art. For transformation of dicotyledonous species using binary *Agrobacterium* vectors such as pCIB200 see Alexander *et al., Proc. Natl. Acad. Sci. 90:* 7327-7331 (1993), and for transformation of monocotyledonous species using direct gene transfer vectors such as pCIB3064 see Koziel *et al., Biotechnology 11:* 194-200 (1993). Transgenic plants are screened for high-level expression of the appropriate cDNA by Northern or Western analysis. Plants which express high levels of the gene product are found to have enhanced resistance to plant pathogens.

Other promoters are suitable for the expression of these cDNAs in transgenic plants. These include (but are not restricted to) constitutive promoters (such as those from the ubiquitin and actin genes) and cell and tissue-specific promoters.

For genes involved in the signal transduction of SAR which may cause negative regulation of the SAR pathway, increased disease resistance can be achieved from the constitutive expression of cDNA in antisense to the gene coding sequence. The cloning and transfer of antisense sequences is undertaken in the same way as described above, except that the orientation of the cDNA is inverted to effect expression of antisense transcripts.

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While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the spirit and scope of the present invention.

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EP-A 392 225

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U.S. Patent No. 4,795,855
U.S. Patent No. 4,940,840

WO 87/07299

***DEPOSIT***

NRRL B-21169

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Ciba-Geigy AG
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(H) TELEFAX: +41 61 696 79 76
(I) TELEX: 962 991

(ii) TITLE OF INVENTION:CHEMICALLY REGULATABLE AND ANTI-PATHOGENIC DNA SEQUENCES AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 29

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1079 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 952 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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CATCAATGGG ATGGAATGTA ACGGTGGGAA TTCAGGTGCG GTCAAGGCAA GGATTGGGTA 600

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1250 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 340 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

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(A) LENGTH: 436 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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CGCAGGAACG TAGAGTTCAT CGAGGCGGCG AACCGAGATG GCAGGCTCTC GTACACCCTC 300

GGGGTGAACC AGTTCGCCGA CCTCACCCAC GAGGAGTTCC TTGCCACGCA CACCAGCCGC 360

CGTGTGGTGC CGTCAGAGGA GATGGTGATT ACAACTCGCG CTGGCGTTGT TGTCGAGGGT 420

GCCAATTGTC AGCCGGCGCC AAATGCTGTG CCTCGTAGCA TCAATTGGGT GAATCAAAGC 480

AAAGTCACCC CAGTCAAAAA TCAAGGAAAA GTATGCGGGG CTTGCTGGGC TTTTTCTGCC 540

GTGGCCACGA TCGAAAGCGC CTACGCGGTC GCCAAGCGAG GCGAGCCGCC GGTTCTGTCC 600

GAGCAGGAGC TCATCGACTG TGACACAATC GACAGAGGCT GCACGAGCGG CGAGATGTAC 660

AATGCCTACT TCTGGGTCTT GAGGAACGGC GGCATCGCCA ACAGCTCAAC GTACCCCTAC 720

AAAGAGACTG ACGGCAAGTG CGAGAGAGGG AAACTGCAGG AACACGCGGC CACGATCAGG 780

GACTACAAAT TCGTCAAACC CAACTGCGAG GAGAAGCTCA TGGCAGCCGT GGCGGTGCGA 840

CCCGTCGCCG TCGGGTTCGA CTCCAACGAC GAATGCTTCA AGTTCTACCA AGCTGGTTTG 900

TACGACGGCA TGTGCATCAT GCACGGGGAA TACTTTGGCC CGTGCTCGTC CAACGACCGC 960

ATCCACTCCT TGGCCATTGT CGGGTACGCC GGCAAGGGGG GCGACAGGGT CAAGTACTGG 1020

ATCGCCAAGA ACTCGTGGGG CGAGAAGTGG GGAAAGAAGG GCTACGTCTG GCTGAAGAAG 1080

GATGTTGATG AGCCGGAAGG CCTCTGCGGC CTTGCAATTC AGCCGGTATA TCCTATAGTC 1140

TGATCTGATC TGACGAGATC GACTGCACTG GGCGTGCATG AAACCTACGG AAATGGCATT 1200

CACCTATATT TTGGGTTGCT CTGTATGCAT GGATGCGCCT ACTATATTTT ACTACATATA 1260

TATTCATCTC CCGCTAATAA AACTACATGT CCTTGTATCC ATTTATGCAC GTTTATCCAT 1320

ATCTTGAATA AATTGGATGG ATTGGTTATC CAAAAAAAAA AAAAAAAAAA A 1371

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 723 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAACCGGGAA CCGGAGGCCT CCGACCCCTC ACCTCGCTGT GGTTGACAAC ATCGACCTGT 60

GCCCAAGACC TCGGGGAACG CTACCAACTC CGAACCCAAG TATGTCCCCG CCTTCGACGA 120

GGCCGACGTC AAAGAAGGAG GTGAAGGGCG TTCTGTACCC ACGAGGCGAC GCACGTGTGT 180

CAGTGGAACG GGCAGGCCAG GTCAAACGGC GGGCTCATCG AGGGGATCGC CGACTACGTG 240

CGGCTCAAGG CCGACCTCGC GCCGACGCAC TGGCGCCCGC AGGGGAGCGG CGACCGTTGG 300

GACGAGGGGT ACGACGTGAC GGCCAAGTTC CTGGACTACT GCGACTCCCT CAAGGCCGGG 360

TTCGTGTCGG AGATGAACAG CAAGCTCAAG GACGGATACA GCGACGACTA CTTCGTGCAG 420

ATCCTGGGGA AGAGCGTGGA CCAGCTGTGG AACGACTACA AGGCCAAGTA CCCCCCAGCC 480

CCAGGGCTGA TCGACGATGC ATGCAGTTTG TTGTTGTATG TGTACCGGTC TTCGTCTACA 540

TACAGATACA TTATAGTACT TGTATTACTG TACAATTTAT GTACTGCCTG GAATGGAATA 600

AATCAGCGTT GGCACGGTGT GTGTTAACGA ATTGACGAGA CAAAGGGACC GTCTATAGGT 660

CATGTCATCG GTTGCCTGAA ATACATTGAA CATCATCACT TTCTTTACAG CAAAAAAAAA 720

AAA 723

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 765 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AAATGACAAT TGAAATTTAT TAAAGTAATA TGATCTAATA TGTTCAAACA AGCTCAATCA 60

CAATTTAATT TAATTAATAT TGACCGCGCA TCGCGCGAGT ACGACTACTA GTTCAATTTA 120

GAAAGCTGGA GCCATTGCCG GAGTAATCAT GAAATTGGCG GAGTTGACTT CCGATTCCGG 180

TGATGGTGCG AGTATTCCCG AGCTTTTGAT CTCCGCCATT CCACGGCGTG CATTTCCACC 240

TTGTTCTTCA CACAATCTGG GAAGGTATAC ACCTTCTCCA GCAGCAAGGT TGAAGTAGAG 300

ATCAATAATA TTAGGGCAAT GTTTGTAGCA CTGAGGGGAG CACAGCTTCT GGGTAAAGTG 360

GCACTCGAGG AGAGAATCAG AAGAATTCCG AAAGTATTCC TATCAACACC GCAAGCCTGA 420

ATACACTGGT CGGTTTCAAT CCAGTCTTTG AGCTTATCAG CCTCAATTTC CGACGTCTTA 480

CATGTATATA CTTCTTCACC ACTCCTTTGG AGACGTTTCT CTAACACGCA ACGCTTCCCA 540

GTACTTGAAA TCGCAAAAGC GCATGAGTCC TTGTTTAGAT TCTCGCATGT TATGCTCCCT 600

AGAGTGACTT GAACACAGAA GACAAGTGCA CAAGCAACAA TAGCCAAAGT AAAGTTGTGG 660

AATGAAGCAA CCATGGTGAA AAATCTAGCA ACAATTGATC AGACTATAAA TTTTTTCTGA 720

GTATATATAG CTCCTTTTGG CTTTCGACTC TCCTTTTTTG TGGTT 765

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1054 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GAATTCGGCA CGAGTTTGCG TATACATCAA TACTCCGGCT AAATCGGCAA ATATATCCAA 60

CAAATTAAGC CTTTTAAACC AATCAGTACG TTATCAATAT CATCAATGTC AGATCGTATT 120

GATGTCTTGG CTATGATGAT ATCAGATGAG GAATTTGCAG AGAAACTGCA GATGCAACAT 180

GTTCTCGGAG CCTCTTATGA AAGTTCCCAA GGATTCTGTG GAATTTGCAA TAAGAAAAAG 240

GGAGCAGATC GAATGTTCAA ACTCCAAATC TGCTCTCGTC ATTCTTTCTG TTCTCACTGC 300

ATAGGTCTTT ATGCCCAATC CAAGATTCGC GACAACGTTT TCCCTATAAC TTGTCCAGGT 360

TTGAGATGTC GTGTCGTAAT TCAACCCGAA TCTTGTAGGT CTATCATTCC AGTAAATACA 420

TTTGCAAGGT GGGAAGAGGG CTCGGCCGAG TCAGCTATTC CTAATGGTGA AAAATTCTAT 480

TGTCCTTATA AAAGATGTTC AGGGCTGTTC ATTCATGATC GACATGAAGA GATCGTTCAG 540

TGCATTTGTC CTCTGTGCAA AAAACTGTTC TGTGCGAAGT GCCGAGTCCC TTGGCACACT 600

GGGCGTGATT GTGACAAGTT TCAAAAGGTA GAAAAAGACA GAGAAGATGA GCTAAAACTT 660

AAGCTGCTTG CTGAGATCAA AAAATGGAAG GAATGCCCTA AGTGCAAATC CATTGTGGAG 720

AAGGTTGATG GCTGTATACA CATGACTTGC AGGTGTAAAA TGGAATTTTG CTACGTATGT 780

GGAGGAACGT GGAGCGAAAG GCATTGGAGT TGCCAAGACT AGTTATGATA TACTATCGGA 840

TTGCCTCTTC ATACCAATCG GATGCTGAAT ATTTTGTTGT CAGAATAAAA ATTATGTTGT 900

TTAGTTGGTT GTATTATGTC TTTAATTTGG TGTCCATGGT TTGTATTAGG AGCATTATAT 960

ATAGTACGAG TTGGTCCTTG CATAACATTT ACTTGATGTT GATTATAATA TGCTCGAGTT 1020

GGAGACCCCA AAAAAAAAAA AAAAAAAACG CGAG 1054

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 760 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCGTGCCGC TTTAAGTGTG CTCGAACCTC CTACTGGTAA TGAGGATGAT GATGACCTGG 60

AATTTGAAAA TGTCCACTGG AATGGTTCAG ATATGGCATC CGATGATACT CAAAAATCTC 120

ATAGACCAAG GCACCGCGTA CATAAATCGT CTGGTTCCCA CAAGGCCATG AGCCGCTCCC 180

TTTCATGTGA CTCGCAATCA AAAGGATCTA TTTCTACACC TCGTGGGTCC ATGGTTGACC 240

TAAGCAAACT CGAGATGGCT GCACTGTGGA GATATTGGCG ACACTTTAAC CTTAGGGAAG 300

CTATACCTAA CCCCTCGAAA GAGCAACTAA TTGATGTTGT CCAGAAGCAT TTCACATCTC 360

AGCAATTGGA CGAGTTGCAG GTAATTGTGG GATTTGTTCA AGCTGCCAAG AGACTCAAGA 420

CAGTCTGCAA ATGACTCAAG GGAACGAACC CCTTTTGGGA ACGCCCCTAA CACTAACAAA 480

TAGTGGTACC TTTGTGTCCA CTTGCTATTG CGCAGTAATA TGTGTAGTAT AATATGTATA 540

CTCCTGTTTA TCCCTTATGT CTTGCTTAGA GTTGTAGTTT ATGTAGATAA ATGAACTGCT 600

GAACTAACTC TGGAGGTCTC CTTCTGGTTG AATGTACTTC CTCGCGAGTG GGGGAAGTAC 660

TAGTACTTAA TCACATGTAT AGTCTAATGT CTTAAACTGT TAAATCATCA TTCAACCAAC 720

TGAATCTTGG TTTGGTATTA GTGACCCTCG TGCCGAATTC 760

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGCCGCTTTA AGTGTGCTCG AACCTCCTAC TGGTAATGAG GATGATGATG ACCTGGAATT 60

TGAAAATGTC CACTGGAATG GTTCAGATAT GGCATCCGAT GATACTCAAA AATCTCATAG 120

ACCAAGGCAC CGCGTACATA AATCGTCTGG TTCCCACAAG GNCATGAGCC GCTCCCTTTC 180

ATGTGACTCG CAATCAAAAG GATCTATTTC TACACCTCGT GGGTCCATGG TTGACCTAAG 240

CAAACTCGAG ATGGCTGCAC TGTGGAGATA TTGGCGACAC TTTAACCTTA GGGAGGTATT 300

CCTAACCCTC GAAAGAGCAC TTATTGATGT GGTCAGAGCA TTCANATCTC AGAAATGGGC 360

GAGTGGAGGT ATTGTGGATT GGTCAAGCTG CAAGAG 396

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 653 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTTGTTTGGT TGTTTGAGTT ATTTTGCTTC TAAGAACTTT GTGAGAAATG GCTGCTAACG 60

ATGCTACTTC ATCCGAGGAG GGACAAGTGT TCGGCTGCCA CAAGGTTGAG GAATGGAACG 120

AGTACTTCAA GAAAGGCGTT GAGACTAAGA AACTGGTGGT GGTCGATTTT ACTGCTTCAT 180

GGTGCGGSCC TTGCCGTTTT ATTGCCCCAA TTCTTGCTGA CATTGCTAAG AAGATGCCCC 240

ATGTTATATT CCTCAAGGTT GATGTTGATG AACTGAAGAC TGTTTCAGCG GGAATGGAGT 300

GTGGAGGCAA TGCCAACTTT TGTCTTCATT AAAGATGGAA AAGAAGTGGA CAGAGTTGTT 360

GGTGCCAAGA AAGAGGAGTT GCAGCAGACC ATAGTGAAGC ATGCTGCTCC TGCTACTGTC 420

ACTGCTTGAA TCTCCTTAAT CAAGGGGATG ATATCCCATA TTTAGTAGTA TTGTCTTTTG 480

TAATAACCAA GTAACTTGTT CGAATTTCAC ACTATGGATC ACTGTATGGT TGTACTATCC 540

ACCATGTTTT TATTGCTTTT GTGAACCTTG TCTTGTTGCT TGGAATCTGA TTTGTGCATT 600

ACTGGTGTAA GGCTATATGC CCAATTCYAC AAAAAGACTA CTTTTAGATT TCT 653

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1697 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTCAAAATT ATTGGATCCT AGTTTTAAGT GGAACTCCTA ATTAATCAAT CATTTCATGA 60

TCAACCTTTA CTAACATCAC CATTTTCCTT GACAACATTA TTTTCCGACT TACCATTTTC 120

CTTAACACCA TTACCCTCTG GATTTTCCTT GACAATATTA CTCTCCGACT TCTCATTTTC 180

GTTCACTTTA TCAGTAATCT TGGCTTCTTC AGCAGCTGCT TCTTTCTCTT TCTCTTTCTC 240

TTTCTCAGCC CTTTCTCTTT CTTCTTCCTC AGCCTTCAAT TTTGGCTCCT CCTTTGCAGT 300

TTCAAGAGCT TTAATCAGTC TCTCCAAACA AGTGTTTGCA TTTTCCTTAG AAGACTTGGG 360

CATTAAATTC TCAGCAACAT CAGCAGGAGT AATATTAGTT TCCCCCAATA AATGACGAAT 420

CTCAGGAAAA TGATCATGAG ACTCAATATC TAGATAATTA TTTGCAAGCA CCTTGAAGGA 480

GTCAAAGCAA CAGTATGATA ACACAATGTG TTTATCCATC CTCCCCCTCC GAATTAAAGC 540

AGGGTCAAGC TTTTCCACAA AGTTGGTAGT GAAAACAATA AGCCTTTCAC CACCAATAGC 600

TGACCATAAC CCATCAATAA AGTTCAAAAG CCCAGATAAA GTCACCTCGC TTTGCTTTTT 660

CTCCTCTCGA TTTTTCATCT TCTCCTTGAC GGCATCTTTC TCGTCTTTTA CTTCCTCTTC 720

CTTGTCGTCT TTCTTCTTCT CCCTTTGGCC GGTAAGGTCA AGCGAACAGT CGATGTCTTC 780

AATCACAATG ATAGACTTAC TAGTAGTATC TATTAATAAC TTTCTTAGCT CGGTGTTGTC 840

CTTAACCGCT GTCAATTCAA GATCATAGAC ATCATATTGT AAGAAGTTAG CCATTGCAGC 900

AATCATGCTA GACTTACCGG TTCCTGGAGG ACCATATAGA AGATAACCAC GCTTCCATGC 960

CTTGCCAATC TTGGCATAAT AGTCTTTTGA CTTGCTAAAT GTTTGAAGGT CATCCATAAT 1020

CTCTTGTTTC TTGTTTGGCT CCATGGCTAA AGTATCAAAT GTTGATGGAT GTTCAAACAC 1080

TACTTGGCTC CACATTCTCC TCCTGTATCC ATACCCACCA TCTCCCTTAC TGTTTGTGTA 1140

CAACTTTCTC TGCCTTTCTC TTACTGAAAT TGCCTTCCCT TCGTCCAATA CATACTTCAA 1200

GTATGAAGCG GTGATAAGCT CGCGGTTCTT TCTGTGAAAC TTGAGTTTGA AATACCTCTT 1260

CTCATCCTCC CTAGGGTACC AAGAAATTGT CTGTCTGCTG GCTACTTGTT GGCTAGAAAT 1320

CCACCAGACT TTCTCGCCTT TATATTCATC GGTTACCTCC TCATGATCAT CCATGGTTAG 1380

TACAAGAGAT TGGCCATCTT TCACTACATT GGCTTTGAGA CGCTTAGCTT GTGTGGAGGA 1440

GTTCTTGCTT AGGTACCTTT CAATTGCTAC ATAAGCTTTG CTACGCTCGA ACCAGCCATC 1500

AGTTTCATAC TCATGAAAAA TAATGTGCAT ATAAGGGTAG AAATAGCTCA CGAGTTTATC 1560

GGTATACCTC CTAATATGAC CACGAAGTTC GTGAGGAAAA TAGTTCTGGT ACATGGTCCA 1620

GGCAAACATG ATTGTTGCAA TAGCTGGACC CAACTGAGTC CAAACATCTT GCATCATCAT 1680

CATCATCTAA TTTCTCT 1697

(2) INFORMATION FOR SEQ ID NO: 15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 654 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTAACCTTGA GATAAGCATT AAAAAAACTC AATGGCAGGG AAGGTTGAGA AAGTGCTTGC 60

AGTACTGATG CTTGCAATGC TTCTGTTTTC GGAGCATTTC ATGGCTGCTA ATCATGAAAT 120

TAAAACAACT GAAGATAACT CTATTAGCCC TTTCTGCTTA ATAAAATGTT TATATGGATG 180

CAGGGGGTTG CCACCTGCAA AAGCAGCCAT TTGTGCAGCT CAATGTTTGT TTAAGTGCGC 240

TGTCCAAGAT GAGGCCAATA TAGCTGAAAC TAAGGGCATA ATAGGTGAGA CTGCATACAA 300

CCAGTATGAT GTTGGATGTG CCCTTGGCTA CTGCTCTGAG TTCCTGTTGA ATTATGATGA 360

GAGGAGGTTC AACTGCTGCA TGGAATACTG TCGCGAGGGC AAAATGACCT GTCCTGTTGA 420

GGCTGCACCT TGAAGAAATG GTTGCCCTAA AATTATCGCC TCATCAAATG GAAGTACACT 480

GCTTTTTCTA CTTCCGGTGT TTAGTAGTAG TAGTAAATAA GTGAGGCATG TTACGTACTC 540

TTATGTTTTG TAATAATTAT GCTTTTTAAT AATGTAATCT GTCTGTGTGC ATACAATGCA 600

CACGACGCTA GCTACTACTT TTTATCTACT AAAAACGAAA AGTAACTTAT TTCT 654

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1031 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAGACAAATA CCTATAATAA GCCATTCATA ATCTTCTTGC TTCTTGTTCA GAATAATGGG 60

GAATCTTTTC TGTTGCGTGC TTGTGAAGCA ATCAGATGTT GCGGTCAAGG AGAGATTTGG 120

CAAATTCCAA AAAGTACTTA ATCCAGGTCT CCAATTTGTT CCATGGGTCA TCGGTGATTA 180

CGTCGCCGGT ACACTGACCC TTCGTCTTCA GCAACTCGAT GTTCAGTGTG AAACCAAAAC 240

AAAGGACAAT GTGTTTGTGA CAGTGGTTGC ATCCATACAA TACAGAGTCT TAGCTGACAA 300

GGCAAGTGAT GCTTTTTACA GACTCAGCAA TCCAACCACC CAAATCAAAG CCTACGTCTT 360

TGATGTGATC AGAGCATGTG TTCCAAAGCT GAACTTGGAC GATGTGTTCG AGCAGAAGAA 420

TGAAATTGCC AAATCTGTGG AAGAAGAGCT AGACAAAGCC ATGACTGCTT ATGGTTACGA 480

AATCCTTCAA ACCCTAATTA TCGACATTGA GCCTGATCAA CAGGTTAAAC GTGCCATGAA 540

CGAAATCAAC GCCGCGGCGA GGATGAGAGT GGCAGCGAGC GAAAAAGCAG AGGCTGAGAA 600

AATCATTCAG ATCAAAAGAG CAGAGGGTGA AGCAGAGTCA AAGTACCTGT CGGGACTCGG 660

AATCGCTCGG CAGAGACAAG CGATCGTGGA CGGTCTTGAG AGACAGTGTT CTTGGGTTCG 720

CAGGAAACGT GCCAGGGACG TCAGCGAAGG ATGTGTTGGA CATGGTGATG ATGACTCAGT 780

ACTTTGACAC AATGAGAGAT ATCGGAGCAA CTTCTAAATC CTCTGCGGTG TTTATCCCTC 840

ACGGTCCAGG CGCCGTCTCT GACGTGGCAG CGCAGATTCG AAATGGATTA TTACAGGCCA 900

ACAATGCCTC CTAATCACTC AAGTCAAATT GTCTTGGTCG TCTCTTTATA TATTTTCGTA 960

TCTTCTTATT AAAAAGGTAA ATTTGACTTT TAATATAATG GTGTGCTTAT TGCGAAAAAA 1020

AAAAAAAAAA A 1031

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 860 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGGAACAAAA GCTGGAGCTC CACCGCGGTG GCGCCGCTCT AGAACTAGTG GATCCCCCGG 60

GCTGCAGGAA TTCGGCACGA GCAACTTAGA AAAATGAATT TTACTGGCTA TTCTCGATTT 120

TTAATCGTCT TTGTAGCTCT TGTAGGTGCT CTTGTTCTTC CCTCGAAAGC TCAAGATAGC 180

CCACAAGATT ATCTAAGGGT TCACAACCAG GCACGAGGAG CGGTAGGCGT AGGTCCCATG 240

CAGTGGGACG AGAGGGTTGC AGCCTATGCT CGGAGCTACG CAGAACAACT AAGAGGCAAC 300

TGCAGACTCA TACACTCTGG TGGGCCTTAC GGGGAAAACT TAGCCTGGGG TAGCGGTGAC 360

TTGTCTGGCG TCTCCGCCGT GAACATGTGG GTTAGCGAGA AGGCTAACTA CAACTACGCT 420

GCGAACACGT GCAATGGAGT TTGTGGTCAC TACACTCAAG TTGTTTGGAG AAAGTCAGTG 480

AGACTCGGAT GTGCCAAAGT GAGGTGTAAC AATGGTGGAA CCATAATCAG TTGCAACTAT 540

GATCCTCGTG GGAATTATGT GAACGAGAAG CCATACTAAT GAAGTAATGA TGTGATCATG 600

CATACACACG TACATAAAGG ACGTGTATAT GTATCAGTAT TTCAATAAGG AGCATCATAT 660

GCAGGAYGTA TCAATATTTA TCAAATAATA CAAATAAGAG CTGAGATTAC GAGAATCTAT 720

TTAAATTAAA AGTTACATAC TTAATTATTA TAGTTATATA TGTAAAATAT GTGGCCTTTT 780

TAAAAGTTAC ATAATTAATT ATTATAGTTA ATGTCTTTCA AAAAAAAAAA AAAAAAAACT 840

CGAGGGGGGG CCCGGTACCC 860

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTTGGCGTCT TCCATTTTTC TAAGTTGATA ATGG

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TATCAACTTA GAAAAATGGA AGACGCCAAA

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1064 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AAACAATGAA CATTAAGGTA TCATTACTTT TCATTCTACC AATATTCTTG CTTCTCCTAA 60

CCAGCAAAGT AAAAGCTGGA GATATTGTAG TCTATTGGGG CCAAGATGTA GGAGAAGGTA 120

AATTGATTGA CACATGCAAC TCTGGTCTCT ACAATATTGT CAACATTGCC TTTTTATCTT 180

CTTTTGGCAA TTTCCAAACT CCTAAACTTA ACTTAGCTGG CCATTGTGAA CCATCTTCTG 240

GTGGTTGCCA ACAGTTGACA AAAAGCATCA GACATTGTCA AAGCATAGGC ATTAAAATCA 300

TGCTCTCCAT TGGAGGTGGA ACTCCTACCT ACACATTATC CTCAGTTGAT GATGCCAGAC 360

AAGTTGCTGA TTACCTGTGG AACAATTTTC TCGGCGGCCA ATCATCTTTT AGGCCACTTG 420

GAGATGCTGT ATTAGATGGC ATAGATTTTG ATATTGAACT TGGCCAACCA CATTATATTG 480

CACTTGCCAG GAGACTTTCA GAACATGGCC AACAAGGTAA AAAATTATAC TTAACTGCAG 540

CACCACAATG TCCTTTTCCT GATAAACTTC TTAATGGTGC ATTGCAAACT GGTTTATTTG 600

ACTATGTTTG GGTCCAATTT TACAACAATC CCGAGTGCGA GTTCATGAGC AATTCAGAAA 660

ATTTCAAGAG GAGGTGGAAT CAGTGGACAT CAATCCCTGC AAAGAAGTTG TATATTGGAC 720

TTCCAGCAGC CAAGACAGCC GCGGGTAATG GCTATATTCC AAAGCAAGTG CTAATGTCAC 780

AAGTTTTACC ATTTCTAAAG GGGTCTTCAA AATATGGAGG TGTCATGCTT TGGAATAGAA 840

AATTTGATGT CCAATGTGGC TATAGCTCTG CTATCAGGGG TGCTGTTTAA GTTCTGAATG 900

AACAAGGCGC CCCTGAATCG CTATAAGCCA TCGTTAAGGC CTAAATAAAG CAAGTTAATT 960

TGCTGTTATC TGCCTAGAAA GTACTTAAGT TTTAATTTGT ACTGATGAAA ATGTGAAGGT 1020

CATCTTGTTT CCTTCTTGAT AATAGTAGTA CTATGGTTCT CTTT 1064

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1018 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TGAAGTGTTA GCACACACAC ACAAAAAAAA CTTAAAGTTA TGATCAAATA TAGTTTTCTT 60

TTGACAGCAT TAGTGCTATT TCTTCGAGCA TTAAAACTAG AAGCAGGGGA TATAGTAATA 120

TATTGGGGCC AAAATGGGAA TGAAGGTAGC TTAGCTGACA CTTGTGCAAC AAATAACTAT 180

GCCATTGTCA ATATTGCTTT CCTTGTAGTT TTTGGGAATG GCCAAAATCC AGTGCTAAAT 240

TTAGCTGGTC ATTGTGATCC AAATGCTGGT GCATGCACTG GCTTAAGCAA TGACATTAGA 300

GCTTGTCAAA ACCAAGGCAT CAAAGTTATG CTTTCTCTTG GTGGTGGTGC TGGAAGCTAT 360

TTTCTTTCTT CTGCTGATGA TGCTAGGAAT GTGGCAAATT ATTTGTGGAA CAATTATCTT 420

GGAGGTCAAT CAAACACACG TCCACTAGGA GATGCAGTTC TAGATGGAAT TGATTTTGAT 480

ATAGAAGGCG GGACAACACA ACATTGGGAT GAATTAGCAA AAACTCTATC ACAATTTAGC 540

CAACAAAGGA AAGTATACTT AACTGCAGCT CCACAATGTC CATTCCCAGA TACATGGTTA 600

AATGGGGCAC TTTCCACTGG CTTATTTGAT TATGTTTGGG TTCAATTTTA CAATAATCCA 660

CCGTGTCAAT ACTCCGGTGG GAGCGCGGAC AATTTAAAAA ATTACTGGAA TCAGTGGAAC 720

GCGATTCAAG CTGGAAAAAT TTTTCTGGGA TTGCCAGCAG CTCAAGGAGC AGCTGGAAGT 780

GGTTTTATAC CATCTGATGT TCTTGTTTCT CAGGTTTTAC CATTAATTAA TGGTTCACCA 840

AAGTATGGGG GTGTTATGCT TTGGTCTAAA TTTTATGACA ATGGTTATAG CTCTGCTATT 900

AAGGCTAATG TTTGAGATAT ATGATCATAG CTAGTCAGCT TGTATTAATA TGATGACGTC 960

AATAATGTTA TATTATAAAC TATATAGTAC TCAATAATAA GGCTTTGAAA GTTACTTA 1018

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGGATCCCTG CA 12

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

ATAGTCTTGT TGAGAGTT 18

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TCACGGGTTG GGGTTTCTAC 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AGGAGATGGT TTGGTGGA 18

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ATACGTTCTA CTATCATAGT 51

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

His Phe Cys Tyr Ile Glu Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

Ile Arg Ala Ile Asn Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 731 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:40..528

(D) OTHER INFORMATION:/note= "PR-1mz cDNA clone"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GAATTCCGGG AACTAGTAAT TATCAGCAAA CAACAAACAA TGGCACCGAG GCTAGCGTGC 60

CTCCTAGCTC TGGCCATGGC AGCCATCGTC GTGGCGCCGT GCACGGCCCA GAACTCGCCG 120

CAGGACTACG TGGACCCGCA CAACGCGGCG CGCGCCGACG TGGGCGTCGG GCCGGTGTCC 180

TGGGACGACA CCGTGGCCGC GTACGCGCAG AGCTACGCGG CGCAGCGCCA GGGCGACTGC 240

AAGCTGATCC ACTCCGGCGG GCCCTACGGC GAGAACCTCT TCTGGGGCTC CGCCGGCGCC 300

GACTGGTCGG CGTCCGACGC CGTGGGCTCC TGGGTGTCCG AGAAGCAGTA CTACGACCAC 360

GACACCAACA GCTGCGCGGA GGGGCAGGTG TGCGGCCACT ACACGCAGGT GGTGTGGCGC 420

GACTCCACCG CCATCGGCTG TGCCCGCGTC GTCTGCGACA ACAACGCCGG CGTCTTCATC 480

ATCTGCAGCT ACAACCCGCC GGGCAACGTC GTCGGCGAGA GCCCCTACTA GACTGTCATG 540

CATACTACAA TTATATATAT ATACGCTTAA TATTAACTTC AGCATGCATG CATATTATAA 600

ATAGTGTTGT CAACTCTGTA TATCATTATA TTACGATGAT TATATTGTTG ATGAACATAA 660

TAAGTCAATA AAACCATGCA GGCGATTTGT GAGCCAAAAA AAAAAAAAAA AAAAAAAAAA 720

AAAAACTCGA G 731

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 163 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Met Ala Pro Arg Leu Ala Cys Leu Leu Ala Leu Ala Met Ala Ala Ile
1 5 10 15

Val Val Ala Pro Cys Thr Ala Gln Asn Ser Pro Gln Asp Tyr Val Asp
20 25 30

Pro His Asn Ala Ala Arg Ala Asp Val Gly Val Gly Pro Val Ser Trp
35 40 45

Asp Asp Thr Val Ala Ala Tyr Ala Gln Ser Tyr Ala Ala Gln Arg Gln
50 55 60

Gly Asp Cys Lys Leu Ile His Ser Gly Gly Pro Tyr Gly Glu Asn Leu

65 70 75 80

Phe Trp Gly Ser Ala Gly Ala Asp Trp Ser Ala Ser Asp Ala Val Gly
85 90 95

Ser Trp Val Ser Glu Lys Gln Tyr Tyr Asp His Asp Thr Asn Ser Cys
100 105 110

Ala Glu Gly Gln Val Cys Gly His Tyr Thr Gln Val Val Trp Arg Asp
115 120 125

Ser Thr Ala Ile Gly Cys Ala Arg Val Val Cys Asp Asn Asn Ala Gly
130 135 140

Val Phe Ile Ile Cys Ser Tyr Asn Pro Pro Gly Asn Val Val Gly Glu
145 150 155 160

Ser Pro Tyr

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 831 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:72..587
(D) OTHER INFORMATION:/note= "PR-5mz cDNA clone"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GAATTCCGGA GCCTAGCTCT ATAGCTCGAG TATTGCTTGC TCTCCACAAG AAACAAGGCA 60

GAGCACCAAC AATGGCCGCC GCGTCCTCGG TCCTCCTGCT GCTCCTGGCC GCCGCCTTGG 120

CTGGCATGAG CGCCAACGCC GCCACCTTCA CCATCACCAA CAACTGCGGG TTCACCGTGT 180

GGCCCCGCGGC CACCCCGGTG GGCGGGGGCA CGCAGCTGAA CCCGGGCGGG ACGTGGACCG 240

TCAACGTGCC GGCCGGCACC AGCTCCGGCC GCGTGTGGGG CCGCACCGGC TGCTCCTTCA 300

ACGGCAACAG CGGGAGCTGC CAGACGGGCG ACTGCGGCGG CGCGCTGGCC TGCACGCTGT 360

CGGGGCAGCC GCCGCTGACG CTGGCCGAGT TCACCATCGG CGGCAGCCAG GACTTCTACG 420

ACATCTCGGT CATCGACGGC TACAACCTCG CCATGGCCTT CTCCTGCAGC ACCGGCGTGC 480

GGCTGGTGTG CACGGACCCT GGATGCCCCG ACGCGTACCA CAACCCCCCC GACATGAAGA 540

CCCATGCCTG TGGCGGCAAC AGCAACTACC AAGTCACCTT CTGCCCGTGA TCGGCCGGAA 600

TAGGCTCTGC ATGCATGGGC TAGTGATTTG GATTTTGCAG GAATAATTCT GATTTGGATT 660

TGCACAATAA TATATAAGCG TGCATTACAC GTGCACGCAT TTGTATGTAC GCGCGTATAC 720

GTCGTACCTA CGTAGCTGGA TTTGTGTGCG TGCTTCGTAC CAAATATTAA TAAGAATAAA 780

CAAATATGAC TCGTGTCATA TATATATAAA AAAAAAAAAA AAAAACTCGA G 831

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 172 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Met Ala Ala Ala Ser Ser Val Leu Leu Leu Leu Ala Ala Ala Leu
1 5 10 15

Ala Gly Met Ser Ala Asn Ala Ala Thr Phe Thr Ile Thr Asn Asn Cys
20 25 30

Gly Phe Thr Val Trp Pro Ala Ala Thr Pro Val Gly Gly Gly Thr Gln
35 40 45

Leu Asn Pro Gly Gly Thr Trp Thr Val Asn Val Pro Ala Gly Thr Ser
50 55 60

Ser Gly Arg Val Trp Gly Arg Thr Gly Cys Ser Phe Asn Gly Asn Ser
65 70 75 80

Gly Ser Cys Gln Thr Gly Asp Cys Gly Gly Ala Leu Ala Cys Thr Leu
85 90 95

Ser Gly Gln Pro Pro Leu Thr Leu Ala Glu Phe Thr Ile Gly Gly Ser
100 105 110

Gln Asp Phe Tyr Asp Ile Ser Val Ile Asp Gly Tyr Asn Leu Ala Met
115 120 125

Ala Phe Ser Cys Ser Thr Gly Val Arg Leu Val Cys Thr Asp Pro Gly
130 135 140

Cys Pro Asp Ala Tyr His Asn Pro Pro Asp Met Lys Thr His Ala Cys
145 150 155 160

Gly Gly Asn Ser Asn Tyr Gln Val Thr Phe Cys Pro
165 170

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 790 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..790
(D) OTHER INFORMATION:/note= "cDNA called PSI-1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GGCACGAGGT CATATTCGTT GAAAACATCA TTCACTCTTC TCTGTGTCTC CTTCTTTCAC 60

CCGAAATTGT CTCTGACACC GNCTTGNTNC AGNCATGGCT AATGCTGCGT CAGGAATGGC 120

AGTCCATGAT GACTGCWWRY KARKAKYYCY KGGAMTGAAG GCGAAAAGGA CACAMCRYTT 180

CATTGTCTAC AAGATTGAGG AGAAGCAGAA GCAAGTGATT GTTGAGAAAG TTGGTGAACC 240

TATTCTAACT TACGAGGACT TTGCAGCAAG TCTTCCAGCT GACGAATGMC GATACGCCAT 300

YTATGATTTC GACTTTGTCA CTGCAGAGAA TTGCCAGAAG AGCAAGATTT TCTTCATTGC 360

ATGGTGTCCC GACGTAGCAA AGGTGAGAAG CAAGATGATC TATGCGAGCT CTAAGGACAG 420

GTTCAAGCGT GAACTTGATG GAATTCAAGT GGAGCTTCAA GCAACTGATC CAACTGAGAT 480

GGATCTTGAT GTTTTGAAAA GCCGCGTCAA CTAAAAGAAA CTCTCTTTGA ATAAGCTTCC 540

GATTATATTC GACTATTATG AAATGGTGTT TTCTATTCTC TTACAGTCTC TTGTGACTGT 600

TAGATACACT TGACACCCTT CATTCTATCT GTCTGGTCTG TTCTCTGTGT CTATGTTACC 660

TTACAGTGTC TCATCTATAT TCAAAATTAT CACAAGGTTT GTGCTGTGTT TGCTTCCTTT 720

GTTTCAACTC TACCAAAGCT TAAGATTAAG ATTTGGGTAA GATGATTCTT TTAAAAAAAA 780

AAAAAAAAAA 790

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 508 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..508
(D) OTHER INFORMATION:/note= "cDNA called PSI-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CCTCCAAGAA CCTAGTGGGA TCCCCCCGGC CTGCAGGAAT TCGGCACCGA GCTCCTTGCC 60

GAAATTCGGC ACGAGTGTTG ATCTAAATCC TCACTAGGTG GTTCCGTTTA AGCTTAGCAA 120

CGCCAAAGAT CTAGACAATA ATTGCAAGTT ACCGCGTTGA TCCTATCTTC CGTCCCGAAG 180

CCGTCCTTGA TCCAGCCCAA GATGTTTTGT GGTCAGATGA CAAAGTAGAT CCACGGGTAT 240

ATATTATGGT AAAGTTGGCG GCCGAAGGAT TGAAGTTCAA TAATGATATG TTTTCTGGTG 300

GTTGCATCCC ATCTGAAGTG GTTATAGCCC CCAAAAACAA AAAATCTCTA CAATTTTGAA 360

AGGTGTTCGT GGCAGGAAAT CTGCTAAGCA AGGAAGGATC TCCAAGAATC CAACTGGGAA 420

TAGAAGGATC CATCAATCAT TCTCTAATGA GGAGGAATCT GTTTAACTTT GTTATTGACT 480

AATAATCCTA TTATCGCCTA AAGCTGTT 508

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 570 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..570
(D) OTHER INFORMATION:/note= "cDNA called PSI-3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GGCACGAGCT CGTGCCGCTC GTGCCGAATT CGGCACGAGG CAACATTTGC AACACCATCG 60

ACGGTGATAG GCCTCGGAGG ATCATCCATC ACCACCAAAC CCTTCTCTTC ATCCTTTTTA 120

AAACCAACAT TAAGCGCCAA GAACCCTTTG AGACTCGCCG GTGCATCGGG AGGAAGAGTC 180

ACTTGCTTTG AGAGGAACTG GTTGAGGAGA GATTTGAACG TGGTAGGATT TGGGCTGATC 240

GGATGGCTAG CTCCGTCGAG CATTCCAGCG ATAAATGGGA AGAGCCTGAC GGGTCTCTTC 300

TTCGATAGCA TCGGAACTGA GCTCGCTCAC TTCCCGACTC CTCCAGCTCT CACTTCACAG 360

TTCTGGTTGT GGTTGGTTAC GTGGCACTTA GGCCTCTTCC TCTGCCTCAC TTTCGGACAA 420

ATCGGATTCA AGGGCAGGAC TGAAGATTAC TTCTAAGGAT AACTATTCTT GTTTTCGTTT 480

GTACTATATG CTCTCTCTGG KTATGTGTAA TATTATCAAT CAAAACAAAG CTTTCTTTGC 540

TATTTGATGT TTCATTCTAT AAAAAAAAAA 570

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1418 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..1418
(D) OTHER INFORMATION:/note= "cDNA called PSI-4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GGCACGAGCA GAATCCTTAG CATCAGAGAA TTCTTCAATG ACAGACACTT ATAACCAGCA 60

GAGAGGTCTT GTCAACCAAC TAAAAGATGA CATGGAGAGG CTATATCAAC AGATCCAAGC 120

CCAAATGGGC GAACTTGAGT CTGTCAGGGT TGAGTATGCA AACGCGCAGC TAGAATGTAA 180

TGCTGCTGAT GAGCGTTCCC AAATACTGGC TTCTGAAGTC ATCAGTTTGG AAGATAAGGC 240

TCTCAGACTC AGGATCTAAT GAGTTAAAGC TGGAGAGGGA ACTGGAGAAG GCACAAAACA 300

GAAATTGTTA TCTTACAAGA AAAAATTGCA GGAGCTTAGA GAAAGGATCC GTCAAAGATT 360

TACAAATCTT ACTATTAAAG CTCTTCAGGA AAGAGAAAGG AAGGGTCCCT ACAAAACTAT 420

GGGTGCAGGA AAGCTTCATC TGGTGGGGAA AAATCCACTG GATCTTTGAG TTAAAACTCA 480

ACTAGCAGAA AGAACGTGTC AACCTCTACA GAAGGTCTTG CAATCTCAGA TACTACGCCA 540

GAGAGCTCCA ACCAGGAAAC AGATTCTACT ACTCTGCTCG AAAGTGATTC ATCTWATACA 600

GCTATCATTC CTGAAACTAG ACAATTAACT CTTGAAGGCT TTTCATTGAG CGTCCCAGCT 660

GATCAGATGA GAGTGATTCA TAACATTAAT ACGCTGATTG CTGAGTTGGC AATTGAGAAG 720

GAAGAACTGG TGCAAGCACT GTCATCTGAG TTATCTCGAA GTGCGCATGT GCAGGAGCTG 780

AACAAAGAGT TATCCAGAAA ACTTGAAGCG CAGACGCAAA GGTTAGAGCT TGTAACAGCG 840

CAGAAGATGG CCATAGACAA TGTTTCACCA GAAAAGCAGC AGSCGGACAC TCATGTTGTT 900

CAAGAGAGAA CMCCGATTGC AGATGAAGGC GATGAGGTGG TAGAAAGGGT TCTWAGGATG 960

GATCATGAAG ATGTTCCCAG GAGGACCGTC GAAAAGAAGG ACAAGCAAGC TTCTCTAATG 1020

CATGGATGGC TTGCTCAGTC GTGACCCTTG TTGCCATTCT GTATATCCCG GGCACAGCCA 1080

TGGATTGCTT CTTCTCCTTT AGKKKTGGAA GAATTCATTT GGTTCTTTTG ATCCCCAAAT 1140

TTTGGATTTC ATTGGATTGA GCMCAAATGG TATAGTGAGA AACACCATGA ACTTGAAGAG 1200

TTCCAAATGG ATAGAGGAGA CTCGGAAGCT TAAACAGAGA GGAAGGTAGT TGTTATATGA 1260

GTGCGTTTTT GTCTGAATTT TGTAATTGTT GAATATATAG CCAAGTTAAA GTGTTCCTCC 1320

TAGCAAATAT AATTTACCAA GAAAAAATCT TGTATGGTTT CGGATATATT TTATCTTCTC 1380

GTTTTAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAA 1418

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 913 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:1..913
(D) OTHER INFORMATION:/note= "cDNA called PSI-5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GGCACGAGAG ACAATCAGAT TCTTTACTCT TACAACGGTG GAGACCAAAC CAACGAGAGT 60

CTTGCTGCTC TGTGTTTAGA AAAGTCTCCT CCTTTTCATA CTTGGTACTT TGAAACTATT 120

GGTAAAAGAA GATTTGGGTT TTTAATTGGA GATGGGTTTG TTTATTTCGC CATTGTTGAT 180

GAGGTTTTGA AGAGATCTAG TGTTCTTAAG TTTCTTGAAC ATTTGGAGAG ATGAATTTAA 240

GAAAGCTGCT AGGGAGAATT CTAGAGGAAG TTTCACTGCT ATGATTGGTT CTATTAATGT 300

TGAAGATCAG CTTGTTCCTG TTGTTACTAG ACTTATAGCT TCTCTTGAAC GTGTTGCTGA 360

GAGTAGTAGT AACAATGAAT TGAAGAGTAG TAATCTTGGT GAACAAAGCG AAGGTTCGAA 420

TTCTACTAAA GCTCCGTTGT TGGGGAGGTT AAGTAAGCAA GAGAAGAAGA AAGGGAAAGA 480

TCATGTGATT GAGCTTGAGG AACACAGGAA GTCTAATGAT AGAGGGAACA TAACCGATGA 540

TTCAGCGGGA GCTGGAACGT CGTTGGAGAA GGAGTGTGTG TCGAGTAGTG GACGTTCTGT 600

TACTCAAAGC TTTGAATGGA AATGGCGGCG GYTAGTTCAG ATTGTTCTTG CTATTGATGC 660

AGCTATTTGC TTGACACTGT TTGGTATTTG GCTGGCTATA TGTCGGGGTA TCGAGTGTAC 720

ACGTTCGTGA TTGTGCAGAC AGAAAATCGG ATAAACAGAG TCGATTCTTT ACATTCCCTT 780

GTGCATTCCA ATAGCCAAAA AATATGTTGA TTGTAATGTG AACCATTCTT AAGTTATAAT 840

TTGCCTTATG TATTGCTTTA GTCACAGCTT TTGAAATATG TACAGTCGGA TTCTAGAAAA 900

AAAAAAAAAA AAA 913

WHAT IS CLAIMED IS:

1. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13), wherein the wild-type gene corresponding to said SAR gene can be chemically induced in a plant in a protein-synthesis independent manner.

2. A DNA molecule according to claim 1, wherein the corresponding wild-type gene can be chemically induced in a plant despite the presence of the protein synthesis inhibitor cycloheximide.

3. A DNA molecule according to claim 1, wherein the corresponding wild-type gene can be induced in a plant by salicylic acid, isonicotinic acid or methyl benzo-1,2,3-thiadiazole-7-carboxylate.

4. A cDNA molecule according to claim 1.

5. A DNA molecule according to claim 1 comprising DNA selected from the group of DNA molecules p11.30.13 (SEQ ID NO. 11), p66B1 (SEQ ID NO. 14), pDPA2 (SEQ ID NO. 16), PSI-1 (SEQ ID NO. 34), PSI-2 (SEQ ID NO. 35), PSI-3 (SEQ ID NO. 36), PSI- 4 (SEQ ID NO. 37), and PSI-5 (SEQ ID NO. 38).

6. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) obtainable by a method comprising

   (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
   (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
   (c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

7. A recombinant or chimaeric DNA molecule comprising a plant SAR gene different from the DNA molecule p1.4.3 (SEQ ID NO. 12 / SEQ ID NO. 13) obtainable by a method comprising

   (a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
   (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
   (c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

8. A recombinant or chimaeric DNA molecule comprising a plant SAR gene, wherein the wild-type gene corresponding to said SAR gene can be chemically induced in a plant in a protein-synthesis dependent manner.

9. A DNA molecule according to claim 8 wherein the corresponding wild-type gene can only be chemically induced in the absence of the protein synthesis inhibitor cycloheximide.

10. A DNA molecule according to claim 8 wherein the corresponding wild-type gene can be induced by salicylic acid, isonicotinic acid or methyl benzo-1,2,3-thiadiazole-7-carboxylate.

11. A cDNA molecule according to claims 8.

12. A DNA molecule according to claim 8 comprising DNA selected from the group of DNA molecules p1.1.1 (SEQ ID NO. 9), p.11.31.4 (SEQ ID NO. 10), and p.14.22.3 (SEQ ID NO. 15).

13. A recombinant or chimaeric DNA molecule comprising a plant SAR gene obtainable by a method comprising

   (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;

(b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

(c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

14. A recombinant or chimaeric DNA molecule comprising a plant SAR gene obtainable by a method comprising

(a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;

(b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

(c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

15. A recombinant or chimaeric DNA molecule comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a plant.

16. A DNA molecule according to claim 15 wherein the corresponding wild-type gene can be induced in a plant by methyl benzo-1,2,3-thiadiazole-7-carboxylate.

17. A DNA molecule according to claim 15 comprising DNA selected from the group of DNA molecules WCI-1 (SEQ ID NO. 3), WCI-2 (SEQ ID NO. 4 / SEQ ID NO. 5), WCI-3 (SEQ ID NO. 6), WCI-4 (SEQ ID NO. 7), and WCI-5 (SEQ ID NO. 8).

18. A recombinant or chimaeric DNA molecule encoding Arabidopsis class IV chitinase, Tobacco chitinase/lysozyme, Maize PR-1, or Maize PR-5.

19. A DNA molecule according to claim 18 encoding Arabidopsis class IV chitinase type A or B.

20. A DNA molecule according to claim 18 comprising the DNA sequence pChit4-TA (SEQ ID NO. 1), pChit4-TB (SEQ ID NO. 2), pBSCL2 (SEQ ID NO. 20), or pBSTCL226 (SEQ ID NO. 21).

21. A DNA molecule according to claim 18 comprising the DNA sequence PR-1mz (SEQ ID NO. 30), or PR-5mz (SEQ ID NO. 32).

22. A recombinant or chimaeric DNA molecule comprising the promoter region of the Arabidopsis PR-1 gene.

23. A DNA molecule according to claim 18 comprising 4.2 kb upstream of the start codon of the PR-1 wild-type gene.

24. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis independent manner, comprising
    (a) cloning genes which are chemically inducible by differential screening of cDNA libraries;
    (b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and
    (c) identifying clones which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

25. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis independent manner, comprising

    (a) cloning genes which are chemically inducible in the presence or absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;
    (b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

(c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

26. A method according to claims 24 or 25, wherein cycloheximide is used as the protein synthesis inhibitor.

27. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis dependent manner, comprising

(a) cloning genes which are chemically inducible by differential screening of cDNA libraries;

(b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

(c) identifying clones which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

28. A method for obtaining a recombinant or chimaeric DNA molecule encoding a gene which corresponds to a wild-type gene which can be chemically induced in a protein-synthesis dependent manner, comprising

(a) cloning genes which are chemically inducible in the absence of a protein synthesis inhibitor by differential display of isolated RNA using polymerase chain reaction;

(b) further analyzing the amplification products obtained in step (a) by Northern hybridisation to RNA isolated from cells chemically induced in the presence or absence of a protein synthesis inhibitor; and

(c) identifying amplification products which hybridise with RNA chemically induced in the absence of a protein synthesis inhibitor but not with RNA chemically induced in the presence of a protein synthesis inhibitor.

29. A method according to claims 27 or 28, wherein cycloheximide is used as the protein synthesis inhibitor.

30. A method for obtaining a recombinant or chimaeric DNA molecule comprising the cDNA of a wild-type wheat gene which wild-type gene can be chemically induced in a wheat plant, comprising

(a) cloning genes which are chemically inducible by differential screening of wheat cDNA libraries;
(b) further analyzing the clones obtained in step (a) by Northern hybridisation to RNA isolated from wheat cells chemically induced; and
(c) identifying amplification products which hybridise with both RNA chemically induced in the absence of a protein synthesis inhibitor and RNA chemically induced in the presence of a protein synthesis inhibitor.

31. A method for obtaining a recombinant or chimaeric DNA molecule encoding plant class IV chitinase comprising amplifying by the polymerase chain reaction DNA molecules from plant DNA using 5' and 3' oligonucleotides designed degenerate for the peptides HFCYIEE (SEQ ID NO. 27) and IRAING (SEQ ID NO. 28), respectively.

32. A method for obtaining a recombinant or chimaeric DNA molecule encoding plant chitinase/lysozyme comprising screening a plant cDNA library with a cDNA probe encoding cucumber chitinase/lysozyme.

33. A method for obtaining a recombinant or chimaeric DNA molecule comprising the promoter region of the Arabidopsis PR-1 gene comprising
(a) screening of an Arabidopsis cDNA library with a probe of Arabidopsis PR-1 c-DNA; and
(b) identifying a clone carrying the promoter fragment by hybridisation to an oligonucleotide corresponding to coding sequences of the Arabidopsis PR-1 gene.

34. A method of improving protection of a plant against a pest comprising transgenically expressing in said plant two or more DNA molecules encoding anti-pathogenic proteins, wherein the transgenically expressed proteins excert a synergistic effect.

35. A method according to claim 34, wherein two or more DNA molecules according to any one of claims 1-19 are transgenically expressed.

36. A method of protecting plants against pests comprising transgenically expressing in said plant class III chitinase.

37. A method according to claim 36, wherein tobacco plant chitinase III is transgenically expressed.

38. A transgenic plant and the progeny thereof comprising a DNA molecule according to any one of claims 1 to 23 displaying anti-pathogenic properties.

39. A transgenic plant according to claim 38 expressing transgenic Tobacco chitinase/lysozyme.

40. A transgenic plant and the progeny thereof comprising two or more DNA molecules encoding anti-pathogenic proteins, which plant displays synergistic anti-pathogenic properties.

41. A transgenic plant according to claim 40 comprising two or more DNA molecules according to any one of claims 1-23.

42. Seed of a transgenic plant according to any one of claims 38-41.

43. Use of a DNA molecule according to any one of claims 1 to 7 to achieve expression of a protein-synthesis independent plant SAR gene in transgenic plants.

44. Use of a DNA molecule according to any one of claims 8 to 14 to achieve expression of a protein-synthesis dependent plant SAR gene in transgenic plants.

45. Use of a recombinant or chimaeric DNA molecule according to any one of claims 18 to 21 to obtain transgenic plants displaying anti-pathogenic properties.

46. Use of the promoter region according to claim 22 to chemically induce a gene in operable linkage with said promoter.